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Cellular Actions of Neuropeptide Y in the Rat Hippocampus

by

Adam Rory McQuiston ©

A thesis submitted to the Faculty of Graduate Studies in partial
fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Pharmacology

Department of Pharmacology
Edmonton, Alberta
Fall 1995

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Date submitted: 28/6/95

"you been scourin your conscience

and rakin through your memories

but that was the river

this is the sea"

Mike Scott

Universty of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Cellular Actions of Neuropeptide Y in the Rat Hippocampus** submitted by **Adam Rory McQuiston** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Pharmacology**.

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To my parents Iain and Fiona, and my brother Sandy

Abstract

Previously, NPY has been shown to potently inhibit excitatory synaptic transmission onto CA1 and CA3 pyramidal cells of the rat hippocampus via the activation of a Y_2 receptor. Although the dentate gyrus of the hippocampus appears to contain the largest concentration of NPY, it does not appear to affect synaptic transmission in this area. The present studies were undertaken to investigate the site and mechanism of NPY's actions in the rat hippocampus.

In vivo studies in CA3 region of the rat hippocampus previously suggested NPY may activate postsynaptic sigma receptors to potentiate N-methyl-D-aspartate (NMDA) receptors. I have demonstrated that NPY did not modulate responses to iontophoretically applied NMDA but did inhibit evoked synaptic excitation onto CA3 pyramidal cells of rat hippocampal slices. Therefore, NPY does not directly modulate the NMDA receptor via the activation of a sigma receptor.

To more directly examine the site and mechanism of NPY's inhibition of excitatory synaptic transmission in area CA3 of the rat hippocampus, I studied the effect of NPY on spontaneous (sEPSC) and miniature excitatory postsynaptic currents (mEPSC) in CA3 pyramidal neurons. NPY and the Y_2 receptor agonist [ahx$^{5-24}$]NPY decreased the frequency, and in some cases the amplitudes, of sEPSCs. NPY affected neither the frequency nor the amplitude of mEPSCs. A quantal analysis suggested that NPY presynaptically inhibits activity-dependent release of glutamate onto CA3 pyramidal neurons, via the activation of a Y_2 receptor.

NPY receptors in the dentate gyrus appear to be of a different pharmacological subtype (Y_1) than in other areas within the hippocampus. I studied the effect of NPY on

basal intracellular Ca^{2+} ($[Ca^{2+}]_i$) and Ca^{2+} influx in rat dentate granule cells (DGC). NPY inhibited depolarization-induced increases in $[Ca^{2+}]_i$ while not affecting resting $[Ca^{2+}]_i$ using intracellular Ca^{2+} imaging of DGCs in rat hippocampal slices. Whole cell patch clamp recordings from acutely dissociated rat DGCs demonstrated that NPY inhibited voltage-dependent N-type Ca^{2+} channels, predominantly via the activation of a Y_1 receptor, in a Ca^{2+}-insensitive manner.

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List of Abbreviatons

ACH	acetylcholine
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
AP	action potential
ATP	adenosine triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid
$[Ca^{2+}]_i$	intracellular calcium concentration
CA	cornus ammon region of the hippocampus
cAMP	cyclic adenosine 3, 5-monophosphate
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CPON	C flanking Peptide of Neuropeptide Y
CSF	cerebrospinal fluid
CRF	corticotropin-releasing factor
CVS	cardivascular system
DG	dentate gyrus
DGC	dentate granule cell
DRG	dorsal root ganglia
EC	entorhinal cortex
EEG	electroencephalogram

EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tertraacetic acid
ENS	enteric nervous system
EPI	epinephrine
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
5-HT	serotonin
FSH	follicle-stimulating hormone
4-AP	4-aminopyridine
GABA	gamma-aminobutyric acid
GH	growth hormone
GI	gastrointestinal
GTP	guanosine triphosphate
HEPES	N-2hydroxyethylpiperazine-N'2-ethanesulphonic acid
I_{Ca}	calcium current
ICV	intracerebroventricular
IPSP	inhibitory postsynaptic potential
ir	immunoreactivity
K_{Ca}	calcium-activated postassium channel
KS	Kolmogorov-Smirnov
LC	locus coeruleus
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LTP	long term potentiation

mEPSC	miniature excitatory postsynaptic potential
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NE	norepinephrine
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NPY-ir	neuropeptide Y immunoreactivity
NTS	nucleus tractus solitarius
PCP	phencyclidine
PcTX	picrotoxin
PGE$_2$	prostaglandin E$_2$
PP	pancreatic polypeptide
PNS	peripheral nervous system
PTX	pertussis toxin
PVN	paraventricular nucleus of the hypothalamus
PYY	polypeptide YY
REM	rapid eye movement
RVLM	rostral ventrolateral medulla
SCG	superior cervical ganglia
SCN	suprachiasmatic nucleus
sEPSC	spontaneous excitatory postsynaptic potential
sIPSC	spontaneous inhibitory postsyanptic potential
SON	supraoptic nucleus

SRIF	somatostatin
TTX	tetrodotoxin
VIP	vasoactive intestinal peptide
V_m	membrane voltage
ω-CTX-GVIA	ω-conotoxin GVIA

Chapter 1

General Introduction

Neuropeptide Y

Neuropeptide Y (NPY) was originally isolated from porcine brain in 1982 by Tatemoto and colleagues, using a chemical isolation procedure based on the amidation of the C-terminal. Very large yields of NPY were obtained by this isolation procedure indicating that it must be very abundant in the porcine brain (Tatemoto *et al.*, 1982). Based on this abundance, Tatemoto and colleagues speculated that NPY must play an important role in the physiology of the animal. Subsequent to its isolation, NPY has been found to be stored in synaptic granulae (Fried *et al.*, 1985), released upon electrical nerve stimulation (Ekman *et al.*, 1986) and acts at specific receptors (Wahlestedt *et al.*, 1990b), which taken collectively indicates that NPY fits the criteria for a neuromodulator (Wahlestedt and Reis, 1993).

NPY was found to be a 36 amino acid peptide with sequence homology to avian pancreatic polypeptide (PP) and peptide YY (PYY). In fact, at least 50% of the amino acids are identical between these peptides. PYY and NPY are approximately 70% identical. Thus NPY was classified as a member of the family of PP-like peptides. However, given the greater degree of evolutionary conservation of NPY and its wider range of biological activity of NPY compared to PYY and PP, it has been suggested that the family of peptides be called the NPY family (Larhammar *et al.*, 1993). The tertiary structure of NPY has been determined based upon the x-ray crystallographic structure of avian PP and nuclear magnetic resonance of NPY (Allen *et al.*, 1987; Blundell *et al.*, 1981; Glover *et al.*, 1983; MacKerell, 1988; Saudek and Pelton, 1990). The NPY family of peptides all appear to form a hairpin loop. The N-terminal end has a polyproline helix which is connected to an amphiphilic alpha helix by a beta turn. The amidated C-terminus projects away from the hairpin loop. The stable hairpin loop is held together by hydrophobic interactions.

Since NPY's discovery, it has been found in a number of vertebrate and invertebrate species. For example, NPY-immunoreactivity has been found in mammals (Anderson and Reiner, 1990), pigeons (Anderson and Reiner, 1990), lizard (Marti *et al.*, 1990), turtle (Reiner and Oliver, 1987), frog (Danger *et al.*, 1985), newt (Perroteau *et al.*, 1988), bony fishes (Noe *et al.*, 1989; Moons *et al.*, 1989), cartilaginous fish (Vallarino *et al.*, 1988), lamprey (Van Dongen *et al.*, 1985), lobster (*Homerus gammarus)* (Charmantier-Duares *et al.*, 1987), locust (*Locusta migratoria*) (Remy *et al.*, 1988), and the gray fleshfly (*Sarcophaga bullata*) (Schoofs *et al.*, 1988). Furthermore, the amino acid sequence is known for several vertebrates and appears to be highly conserved between species. In fact, NPY at the greatest divergence, is 86% identical in amino acid sequence between mammals and other vertebrates, including elasmobranchs, an evolutionary distance of over 400 million years (Larhammer *et al.*, 1993).

The gene encoding the NPY precursor peptide has been cloned and the structure determined (Minth *et al.*, 1984; 1986). The gene shows 60 % homology between rat and human, and 85 % homology within the open reading frame. The N-terminal is flanked by a 28 amino acid signal peptide which is cleaved early in posttranslational processing. There is no N-terminal flanking peptide in the proNPY peptide. However, there is a 30 amino acid C-terminal flanking peptide called CPON (for C-flanking Peptide Of NPY), which is co-stored and co-released with NPY, but for which no function is currently known (Allen *et al.*, 1987; Suburo *et al.*, 1992). The promoter region contains 5 SP-1 binding sites, two CCCCTC sites, a partial CAAT box and one AP1 binding site (Allen and Balbi, 1993), suggesting that the levels of NPY expression can be regulated by external signals. Interestingly, the AP1 site appears not to be functional (Minth and Dixon, 1990). However, cAMP- and calcium- or phospholipid-dependent protein kinases can regulate NPY expression (Sabol and Higuchi, 1990; Allen and Balbi, 1993).

Given the observation that NPY is found across the entire animal kingdom, and its gene and peptide sequences are stringently conserved across vast evolutionary divergences, it would not be surprising if NPY plays an important role in the physiology and behaviour of animals (see below).

NPY Receptor Subtypes

NPY appears to have at least 3, and possibly 4 different receptor subtypes. The initial observation that NPY receptor subtypes existed came from studies performed on the neuroeffector junction of the mammalian sympathetic nervous system (Wahlestedt *et al.*, 1986; 1987; 1990c; Edvinsson, *et al.*, 1987). This was demonstrated using structure activity studies of NPY, related peptides, C-terminal fragments of NPY, and altered sequences of NPY.

The first evidence for at least two different receptor subtypes for NPY came from studies of sympathetic innervation onto smooth muscle of the vasculature (Wahlestedt *et al.*, 1986; 1987; 1990c; Edvinsson, *et al.*, 1987). NPY released from sympathetic terminals onto vascular smooth muscle has three effects, 1) a direct, postjunctional contraction of the smooth muscle, 2) a postjunctional potentiation of NE-induced smooth muscle contraction, and 3) a prejunctional inhibition of NE and NPY release (Wahlestedt *et al.*, 1990c; Wahlestedt *et al.*, 1986; Wahlestedt *et al.*, 1987; Edvinsson, *et al.*, 1987). Both NPY and PYY are effective at all sites (Wahlestedt *et al.*, 1986), however, removal of a number of N-terminal amino acids from NPY, particularly NPY_{13-36} (ie. NPY with the first 12 amino acids removed from the N-terminal) is only effective on the inhibition of neurotransmitter release with no effect on postjunctional activity (Wahlestedt *et al.*, 1986). Thus the presynaptic, NPY_{13-36} sensitive, receptors were called Y_2 receptors and the postsynaptic, NPY_{13-36} insensitive, receptors were designated Y_1 receptors.

Since the discovery of multiple receptor subtypes for NPY, a selective Y_1 receptor agonist has been developed (Michel, 1991). The agonist is NPY with a leucine substituted at position 31 and a proline substituted at position 34 (Michel, 1991). The substitution of proline at position 34 is the only requirement for Y_1 selectivity. Subsequent studies have shown that the receptor selectivity profile for the Y_1 receptor is $[Pro^{34}]NPY = NPY = PYY >> NPY_{13-36} >> PP$ and the Y_2 receptor is $PYY = NPY > NPY_{13-36} >> [Pro^{34}]NPY > PP$. Indeed the recently cloned Y_1 receptor (Larhammar *et al.*, 1992) has the same potency profile for the agonists listed above.

The selective agonists provide some indication of the requirements for activity at the aforementioned receptor subtypes. The activity of C-terminal fragments on Y_2 but not Y_1 receptors indicates that the N-terminal of NPY is important for activation of Y_1 but not Y_2 receptors (Boublik *et al.*, 1990). Substitution at amino acid 34 does not affect Y_1 receptor activity but reduces Y_2 receptor activity, suggesting the C-terminal structure is more important for the Y_2 receptor (Michel, 1991). The hairpin loop itself does not appear to be important in function of either Y_1 or Y_2 receptor since truncation of the hairpin loop and bridging the N- and C-terminals together, maintains function at both receptor subtypes (Krntenansky *et al.*, 1989; Fuhlendorff *et al.*, 1990; Michel, 1991; Beck *et al.*, 1989). Furthermore, substitution of a proline at position 20, which disrupts the hairpin loop, inhibits activity at Y_1 but not Y_2 receptors (Michel, 1991). This suggests that much of the central part of the molecule does not play a direct role in the activation of the receptor but holds the C- and N-termini close and in position for the activation of the Y_1 receptor.

Not all NPY responses fall into either of the two agonist potency profiles listed above. More specifically, cardiovascular responses to NPY injected into the nucleus of the solitary tract of the rat respond to NPY, NPY_{13-36}, [$Leu^{31}Pro^{34}$]NPY but not PYY (Agnati *et al.*, 1983; Grundemar *et al.*, 1991). The efficacies of NPY_{13-36} and [$Leu^{31}Pro^{34}$]NPY initially suggest that both Y_1 and Y_2 receptors are present, however, the absence of an effect by PYY indicates neither receptor is present. Therefore, according to this agonist profile, a third receptor subtype has been hypothesized to exist called the Y_3 receptor. Similar agonist profiles were seen for inhibition of nicotine-induced release of NE and EPI from the adrenal medulla, supression of I_{Ca} in rat SCG neurons and for binding in cardiac tissue (Bralasubramaniam *et al.*, 1990; Foucart *et al.*, 1993; Li *et al.*, 1990; Sheikh *et al.*, 1989; Wahlestedt *et al.*, 1992). Because PYY and NPY show the greatest divergence in sequence in the hairpin loop and substitutions at the C-terminal and cleaving off portions of the N-terminal does not appear to greatly affect activity at the Y_3 receptor, it would appear that the hairpin loop sequence is important for Y_3 receptor function in constrast to Y_1 and Y_2 receptors.

In the hypothalamus, NPY may be coupled to yet another receptor subtype different from the three mentioned above. NPY when injected into the perifornical hypothalamus elicits a potent increase in feeding in rats (Stanley *et al.*, 1993). The receptor mediating this effect is Y_1-like in that there is little response to NPY C-terminal fragments, where [Pro^{34}]NPY and PYY are at least equipotent (Kalra *et al.*, 1991b; Stanley *et al.*, 1992). However, NPY_{2-36} is even more potent than NPY (Jolicoeur *et al.*, 1991; Kalra *et al.*, 1991a; Leibowitz and Alexander, 1991; Magdalin *et al.*, 1989; Stanley *et al.*, 1992), which is inconsistent with the agonist profile for a Y_1 receptor. Therefore, the feeding response in the hypothalamus may constitute a fourth NPY receptor subtype.

NPY has also been postulated to be the endogenous ligand at the sigma opioid receptor (Roman *et al.*, 1989). Sigma receptors are thought to be a subtype of opioid receptor responsible for some of the psychomimetic effects of ligands such as SKF 10,047 (Keats and Telford, 1964; Snyder and Largent, 1989; Haertzen, 1970). This receptor is insensitive to blockade by the opiate antagonist, naloxone which suggests that it belongs to another receptor subtype. An endogenous peptide ligand has been proposed for this receptor, on the basis that peptide fractions from guinea-pig, porcine and human brain bind with high affinity to sigma or PCP receptors (Quirion *et al.*, 1984; Su *et al.*, 1986) particularly within the hippocampus (Connor and Chavkin, 1991, 1992). Initial evidence that NPY may be the endogenous ligand came from binding studies performed on crude rat brain membranes, which showed that NPY and PYY could bind to sigma receptors with high affinity (Roman *et al.*, 1989). However, these results could not be replicated by other investigators (Quirion *et al.*, 1991; Tam and Mitchell, 1991). Although Quirion and colleagues (1991) could not replicate the in vitro binding of NPY to sigma sites, they did show that NPY, PYY, NPY_{2-36} and [$Leu^{31}Pro^{34}$]-NPY but not NPY_{13-36} could displace 17 to 35 % of SKF 10,047 (sigma ligand) binding in the mouse hippocampus *in vivo* (Bouchard *et al.*, 1993). They have suggested that NPY may modulate the release of an endogenous sigma ligand *in vivo* or NPY may affect sigma ligand binding via a second messenger system since they could only see an effect of NPY on *in vivo* sigma binding (Bouchard *et al.*, 1993) and not *in vitro* (Quirion *et al.*, 1991). The physiology of sigma receptors is also plagued with inconsistencies. In the GI tract, NPY has been shown to inhibit intestinal ion transport (Riviere *et al.*, 1990, 1993), increase bicarbonate transport (Pascaud *et al.*, 1993) and block the inhibitory effects of emotional stress on colonic motility (Junien *et al.*, 1991) all which were inhibited by sigma antagonists. However, in the rat vas deferens, there appears to be no effect of NPY on the contractile response of sigma receptors (Martel *et al.*, 1990; Quirion *et al.*, 1991). NPY has been shown to potentiate NMDA-induced firing of rat CA3 pyramidal neurons *in vivo* (Monnet *et al.*, 1990). This affect was shown to be inhibited by sigma receptor antagonists (Monnet *et al.*, 1990, 1992b). The NPY agonist profile of these NMDA responses was unlike any NPY receptor agonist profile and different from the *in vivo* binding studies of Bouchard *et al.* (1993). NPY, [$Leu^{31}Pro^{34}$]-NPY and NPY_{13-36} (which shows no binding in *in vivo* studies; Bouchard *et al.*, 1993) all enhanced NMDA-induced activation of CA3 pyramidal cells. Interestingly, the inactive C-terminal desamido form of NPY inhibited both NMDA and quisqualate effects on CA3 pyramidal cells where PYY and NPY_{18-36} antagonized the effects of the agonists listed above and NPY_{2-36} (which does show binding *in vivo*; Bouchard *et al.*, 1993). NPY_{11-36} and NPY_{16-36} were without effect (Monnet *et al.*, 1992a). These effects of NPY on CA3 firing were also inhibited by pertussis toxin (Monnet *et al.*, 1994) in contrast to the inhibitory effects of NPY on excitatory synaptic transmission in area CA1 of the rat hippocampus (Colmers and Pittman, 1989). It is interesting to note that Monnet and colleagues (1990, 1992a) did not see a change in the spontaneous firing of CA3 pyramidal cells following application of NPY. This is contrary to what might be expected given the potent inhibitory actions of NPY on excitatory synaptic transmission in both CA1 and CA3 pyramidal cells (Colmers *et al.*, 1985, 1987, 1988; Klapstein and Colmers, 1993). This effect of NPY would be expected to reduce the rate of firing of CA3 pyramidal neurons in the hippocampus by inhibiting the excitatory synaptic transmission which drives these cells to fire action potentials (AP). The effect of NPY and PYY on the NMDA-induced release of NE from rat hippocampal slices has also been examined for sigma receptor effects (Monnet *et al.*, 1991; Roman *et al.*, 1991). Again, inconsistencies arise when these studies are compared to the studies on NMDA-induced CA3 pyramidal cell firing *in vivo* (Monnet *et al.*, 1992a). Both NPY and PYY potentiated NMDA-induced release of NE, which could be blocked by sigma receptor antagonists (Roman *et al.*, 1991). The potentiating effect of PYY in NE release is opposite to the sigma receptor antagonistic effect of PYY on the NMDA induced firing of CA3 pyrmadal cells *in vivo* (Monnet *et al.*, 1992a). Therefore, although NPY has been shown in some studies to act at sigma receptors, there are a great number of inconsistencies which cannot be explained by multiple subtypes of sigma receptors. The role of NPY as the endogenous ligand at the sigma receptor is thus still controversial.

NPY receptors appear to be G-protein coupled receptors and interact with second messenger systems. Indeed, based on hydropathy plots of the amino acid sequence, the Y_1 receptor clone has the predicted seven membrane spanning regions typical of G-protein coupled receptors (Larhammar *et al.*, 1992). Binding of NPY is sensitive to GTP (Feth *et al.*, 1991), indicative of a G-protein coupled receptor. Many of NPY's effects are blocked by pertussis toxin (PTX), a toxin which ADP ribosylates some G-proteins thereby inactivating them. PTX-sensitivity is seen in a variety of cell lines expressing Y_1, (Michel *et al.*, 1990) Y_2 (Wahlestedt *et al.*, 1990b) and Y_3 receptors (Balasabramaniam and Sherriff, 1990), indicating that all three receptor subtypes can be coupled to PTX-sensitive G-proteins. Furthermore, in PTX treated cultured dorsal root ganglion cells, the NPY response can be reconstituted by intracellular application of the alpha subunit of the G_o protein (Ewald *et al.*, 1987) which lends further support to NPY being coupled to PTX-sensitive G-proteins. However, not all NPY responses are PTX-sensitive. The effects of NPY on the release of transmitter in the vas deferens (Michel, 1991), hippocampus (Colmers and Pittman, 1989) and sympathetic nerve terminals of the heart (Foucart and Majewski, 1989) are all insensitive to PTX. Therefore, NPY can be coupled to both PTX-sensitive and -insensitive G-proteins irrespective of the NPY receptor subtype.

NPY receptors can be coupled to a variety of different second messenger systems.

Again Y_1 (Michel *et al.*, 1990), Y_2 (Wahlestedt *et al.*, 1990b) and Y_3 (Balasabramaniam and Sherriff, 1990) receptors can be coupled to the same second messenger system as all three have been shown to inhibit adenylate cyclase in cell lines. NPY has been shown to be coupled to the inhibition of adenylate cyclase in many systems (Fredhom *et al.*, 1985; 1989; Kassis *et al.*, 1987; Krause *et al.*, 1992; Larhammar *et al.*, 1992; Reynolds and Yokota, 1988; Wahlestedt *et al.*, 1990b) such as arteries (Fredhom *et al.*, 1985; 1989; Kassis *et al.*, 1987; Reynolds and Yokota, 1988), vas deferens (Haggblad and Fredholm, 1987), spleen (Lundberg *et al.*, 1988), pancreas (Fredholm *et al.*, 1989), cerebral cortex (Westlind-Danielsson *et al.*, 1987), hippocampus (Fredholm *et al.*, 1989; Petrenko *et al.*, 1987), striatum (Westlind-Danielsson *et al.*, 1987) and medulla oblongata (Monnet *et al.*, 1990). However, NPY may also be coupled to the phospatidylinositol system and intracellular calcium release in some systems. For example, NPY elevates intracellular calcium in vascular smooth muscle (Mihara *et al.*, 1989; Wahlestedt *et al.*, 1992), DRG cells (Perney and Miller, 1989), and erythroleukemia cells (Motulsky and Michel, 1988) and increases phosphatidylinositol turnover in brain (Hinson *et al.*, 1988; Wahlestedt *et al.*, 1987), DRG cells (Ewald *et al.*, 1989; Perney and Miller, 1989; Walker *et al.*, 1988), vas deferens (Haggblad and Fredholm, 1987) and vasculature (Wahlestedt *et al.*, 1990c).

In summary, the known NPY receptors are G-protein coupled receptors, predominantly coupled to PTX-sensitive G-proteins. The NPY receptor subtypes do not appear to be uniquely coupled to specific G-proteins. For the most part, NPY receptors appear to be coupled to inhibition of adenylate cyclase with some cell types showing PI and intracellular calcium responses. Y_1, Y_2, and Y_3 receptors can all couple to the same second messenger systems. However, because the NPY receptor subtypes do not appear to be differentially coupled to any particular G-protein or second messenger system, this makes it more difficult to dissociate the various receptor subtypes, given that at present there are no useful, selective antagonists. Although there appears to be at least three receptor subtypes for NPY, only one has been cloned. As more systems containing NPY and NPY binding are investigated and more clones are discovered, the number of NPY receptors that have been discovered may increase. Also as more systems are investigated, the number of G-proteins and second messenger systems to which NPY receptors can couple may increase.

Distribution of NPY and Its Receptors in the Peripheral and Central Nervous Systems

NPY and its receptors are found widely dispersed and in large concentrations throughout the peripheral and central nervous systems of mammals. The distribution of NPY and its receptors will be first described in the periphery followed by their distributions in the central nervous system of mammals.

NPY has been found to be associated with a number of organ systems, including the gastro-intestinal (GI) system and most dramatically with sympathetic innervation to the neuroeffector junction of blood vessels.

In the vasculature, NPY has been found to be co-localized with norepinpherine (NE) within postganglionic sympathetic nerve terminals (Ekblad *et al.*, 1984; Lundberg *et al.*, 1982 ; 1983; Sundler *et al.*, 1986; Uddman *et al.*, 1985). As the diameter of the blood vessel decreases, the amount of NPY innervation appears to increase. NPY appears to be particularly abundant around cerebral (Cannon *et al.*, 1986; Edvinsson *et al.*, 1987) and coronary arteries (Allen and Bloom, 1986; Ekblad *et al.*, 1984; Uddman *et al.*, 1985). Interestingly, the uterine artery of the guinea pig appears to have two types of afferents, one in which NPY is co-localized with NE and the other which is co-localized with vasoactive intestinal peptide (VIP) (Morris *et al.*, 1985).

A significant amount of NPY is found associated with the GI tract. It is found in the sympathetic terminals innervating of blood vessels and in the enteric nervous system (ENS). In the ENS, NPY has been localized to neurons of the myenteric ganglia of the esophagus (Aggestrup *et al.*, 1987; Keast *et al.*, 1987a; Wattchow *et al.*, 1987; 1988), stomach (Ekblad *et al.*, 1987; Furness *et al.*, 1983; Keast *et al.*,1987; Lee *et al.*, 1985; Wattchow *et al.*, 1987) and NPY has been found in the submucous, myenteric ganglia and blood vessels of the small (Ekblad *et al.*, 1987; 1991; Furness *et al.*, 1983; Keast *et al.*, 1987; Sundler *et al.*, 1983; Timmermans *et al.*, 1990) and large intestine (Ekblad *et al.*, 1988; 1991; Furness *et al.*, 1983; Keast *et al.*, 1987; Wattchow *et al.*, 1988). NPY is often colocalized with CCK, CGRP, somatostatin, dynorphin, enkephalin and VIP (Furness and Costa, 1987), and has been used to define different populations of enteric neurons (Sundler *et al.*, 1993).

NPY has been found in other systems in the periphery as well. NPY has been found in the adrenal medulla (Allen *et al.*, 1983), colocalized with NE and EPI. In the salivary gland, NPY has been found associated with sympathetic nerve terminals that innervate blood vessels (Leblanc and Landis, 1988; Sundler *et al.*, 1989) and parasympathetic nerve terminals which innervate both blood vessels and acini (Hardebo *et al.*, 1992; Leblanc *et al.*, 1987; Leblanc and Landis, 1988; Sundler *et al.*, 1989). NPY has been found in nerve fibers around blood vessels, glandular acini and within smooth muscle of the respiratory system (Hakanson *et al.*, 1983; Lacroix, 1989; Lundberg *et al.*, 1983; 1986; Sheppard *et al.*, 1984, Uddman *et al.*, 1989). Most of these nerve fibers also contain NE, although there is some evidence for nonadrenergic, VIP and NPY colocalized nerve terminals in the airways (Lacroix, 1989; Lundberg *et al.*, 1986). The pancreas appears to contain NPY around blood vessels, ducts, acini in the exocrine parenchyma, and the islets (Sundler *et al.*, 1993). The pancreatic NPY innervation arises from both sympathetic and parasympathetic origins. NPY is present in the urogenital tract including the kidney (Allen *et al.*, 1985) and the urinary bladder (Mattiasson *et al.*, 1985). Interestingly, the kidney appears to have predominantly adrenergic colocalized NPY fibers, in contrast to the urinary bladder, where NPY is found in nonadrenergic fibers. Thus, in these systems of the periphery, NPY can be associated with sympathetic, parasympathetic or other nerve fibers.

NPY is also distributed widely throughout the CNS of mammals. It is found in cells and fibers at every level of the CNS. NPY is expressed by local circuit neurons (ie. inhibitory interneurons) which synapse onto other neurons within the same area and in neurons which project to regions different from its own.

In the cerebral cortex of the rat (Allen *et al.*, 1983), monkey (Beal *et al.*, 1987) and human (Adrian *et al.*, 1983), NPY is found in varying concentrations in different areas of the CNS, with the highest concentrations in the cingulate cortex and in association areas of temporal cortex, whereas low concentrations are found in the frontal lobe and the occipital lobe. The neurons which contain NPY appear to be interneurons of many different types but NPY does not appear to be localized in basket cells (Jones and Hendry, 1989) or chandelier cells (Peters, 1984). They appear to be located in all layers in the cortex, however, they are more concentrated in layers II, II, V and VI, with numerous cell bodies also found in the subcortical white matter (Chan-Palay *et al.*, 1985; Hendry *et al.*, 1984; MacDonald *et al.*, 1982). Like other peptides, NPY is often colocalized with other neurotransmitters or different neuronal markers in CNS neurons (Hokfelt *et al.*, 1980). NPY has been shown to be colocalized with somatostatin (Chronawall *et al.*, 1984; Jones and Hendry, 1986), NADPH-diaphorase (Sharp *et al.*, 1987), GABA, substance P, neurokinin A or SRIF (Aoki and Pickel, 1989). Most NPY fibers do not directly synapse onto a postsynaptic specialization but instead have varicosites which pass in close apposition to many neuronal elements including nerve terminals (Aoki and Pickel, 1990; Hendry *et al.*, 1984).

Like the cerebral cortex, the hippocampus has many NPY immunoreactive interneurons (Chan-Palay *et al.*, 1986; Köhler *et al.*, 1986; Smith *et al.*, 1985). These NPY-ir interneurons have varied morphology, but are predominantly not pyramidal in shape (Köhler *et al.*, 1986). NPY cells are most concentrated in the dentate gyrus (Köhler *et al.*, 1987), however, NPY-ir cells are also found in the strata pyramidale, radiatum, and oriens of Ammon's horn, and the fimbria, alveus and angular bundle fiber tracts of the hippocampus (Blessed *et al.*, 1968; Chan-Palay *et al.*, 1986; 1987; Köhler *et al.*, 1986). Many of the NPY interneurons coexpress somatostatin and GABA (Köhler *et al.*, 1987; Chan-Palay, 1987). NPY-ir fibers are found in the molecular layer of

Ammon's horn and the outer one third of the molecular layer of the dentate gyrus (Köhler *et al.*, 1986). NPY binding is most concentrated in the strata radiatum and oriens of the CA regions and some binding is seen in the molecular layer of the dentate gyrus (Köhler *et al.*, 1987; Lynch *et al.*, 1989; Martel *et al.*, 1986; Schwerdtfeger *et al.*, 1986). Following limbic seizures, NPY is expressed in dentate granule cells (DGC) (Marksteiner *et al.*, 1989).

In the striatum, the caudate nucleus, putamen and nucleus accumbens septi have large amounts of NPY-ir in rat (Allen *et al.*, 1983; Nakagawa *et al.*, 1985), monkey (Khorram *et al.*, 1987) and human (Adrian *et al.*, 1983). Most of the NPY is thought to be contained in the medium aspiny neurons (Kowall *et al.*, 1985; Vincent *et al.*, 1983) colocalized with somatostatin and NADPH diaphorase (Gaspar *et al.*, 1987; Kowall *et al.*, 1985; Smith and Parent, 1986; Vincent *et al.*, 1983). Some NPY cells and nerve terminals also appear to contain GABA (Aoki and Pickel, 1989; Vuillet *et al.*, 1990). The NPY cells have inputs from cortical glutamatergic (Vuillet *et al.*, 1989a,b), nigral dopaminergic (Vuillet *et al.*, 1989a,b) and GABAergic neurons (Aoki and Pickel, 1989; Massari *et al.*, 1988; Vuillet *et al.*, 1990). NPY neurons appear to target a particular subset of GABA neurons which receive sparse inputs themselves (Aoki and Pickel, 1989).

Some of the highest concentrations of NPY-ir in rats (Allen *et al.*, 1983), monkey (Khorram *et al.*, 1987) and humans (Adrian *et al.*, 1983) occurs in the hypothalamus. NPY and its mRNA are found predominantly in the lateral hypothalamus and arcuate nucleus of the rat (Bai *et al.*, 1985; Card *et al.*, 1983). NPY fibers are found throughout the hypothalamus. However, individual fibers have uneven concentrations (Card *et al.*, 1983; Sawchenko *et al.*, 1985). The fibers can be from several origins, for example, the suprachiasmatic nucleus (SCN) is a recipient of input from the lateral geniculate nucleus (Sawchenko *et al.*, 1985), whereas the paraventricular nucleus receives its input from brainstem nuclei (Sawchenko *et al.*, 1985) or the arcuate nucleus (Bai *et al.*, 1985). However, apart from the SCN (Lynch *et al.*, 1989; Martel *et al.*, 1986; Quirion *et al.*, 1990), the hypothalamus appears to contain very little NPY receptor binding, indicating a mismatch (Martel *et al.*, 1988).

NPY has been found to be colocalized with monoamines such as NE, epinephrine (EPI), and serotonin (5-HT). More specifically, NPY and NE have been shown to be colocalized in neurons of the A1 group of the ventrolateral medulla, the A2 group of the dorsal medulla (Blessing *et al.*, 1987; Everitt *et al.*, 1988; Jacobowitz *et al.*, 1982; Lundberg *et al.*, 1980), the locus coeruleus (Everitt *et al.*, 1984; Lundberg *et al.*, 1980; Hunt *et al.*, 1981) and the A4 group of the pons (Everitt *et al.*, 1984). NPY and EPI appear to be colocalized in neurons of the C1 and C2 cell groups (Blessing *et al.*, 1986; Everitt *et al.*, 1988; Halliday *et al.*, 1988; Smialowska, 1988) and the nucleus of the solitary tract (Everitt *et al.*, 1988; Lundberg *et al.*, 1980; Smialowska, 1988).

NPY is also found in other regions of the CNS. NPY-ir is found in the superficial and deep short axons of the deep granule cell layer of the olfactory bulb (Gall *et al.*, 1986; Ohm *et al.*, 1988; Scott *et al.*, 1987), often colocalized with somatostatin and NADPH-diaphorase (Scott *et al.*, 1987). NPY-ir is seen within the septum (Gaspar *et al.*, 1987), basal forebrain (Gaspar *et al.*, 1987), three types of amygdala neurons (Graybiel, 1982), some areas of the thalamus (Dawbarn *et al.*, 1984; Lynch *et al.*, 1989), the central gray, interpeduncular nucleus and inferior colliculus of the midbrain (DeQuidt and Emson, 1986; Morris, 1989), the respiratory brainstem nuclei and the superficial dorsal horn of the spinal cord (Allen *et al.*, 1984; Gibson *et al.*, 1984; Hunt *et al.*, 1981).

Considering the wide distribution of NPY in the periphery and in the circuitry throughout the entire mammalian CNS, many investigators have suggested that NPY may play important roles in the physiology and behaviour of mammals.

Physiological and Behavioural Actions of NPY in the Periphery and CNS of Mammals

In the previous section NPY was shown to be widely dispersed in the periphery and CNS of mammals. In this section, the function NPY in both the periphery and CNS will be described for a number of physiological systems.

Probably the best understood action of NPY arise from its activity on the cardiovascular system (CVS) in the periphery. As discussed above, NPY affects the sympathetic neuromuscular junction in three ways. First, NPY can directly constrict blood vessels by contracting smooth muscle. Second, at concentratons which are subthreshold for a direct effect, NPY can potentiate the contractions elicited by NE and other transmitters in vascular smooth muscle. Lastly, NPY can inhibit the release of both NE and NPY from postganglionic sympathetic nerve terminals (Wahlestedt *et al.*, 1986; 1987; 1990a; Edvinsson *et al.*, 1987). NPY also inhibits the release of ATP, which is colocalized with NE and NPY and is itself a potent vasoconstrictor (Cheung, 1991; Saville *et al.*, 1990). As a general rule, NPY acts via Y_1 receptors postsynaptically and Y_2 receptors presynaptically. The observation that C-terminal fragments (Y_2 selective agonists) cause hypotension is in agreement with this hypothesis (Boublik *et al.*, 1989; Brown *et al.*, 1989; Michel *et al.*, 1990; Scott *et al.*, 1990). However, there are some exceptions, as Y_2 receptors has been found postsynaptically on some vascular smooth muscle (Wahlestedt *et al.*, 1990c).

There are sources of NPY other than sympathetic nerve terminals which may contribute to its actions on the CVS. NPY is colocalized with EPI and NE in chromaffin cells of the adrenal medulla (Allen *et al.*, 1983). However, significant release of NPY from these cells only occurs from pheochromocytomas, adrenal secretory tumors (Corder *et al.*, 1985; Pernow *et al.*, 1986a,b; Morris *et al.*, 1987; Takahashi *et al.*, 1987; Zukowska-Grojec *et al.*, 1988). In rat, NPY has been found in platelets and could contribute significantly to circulating levels of NPY (Ericsson *et al.*, 1987; Myers *et al.*, 1988). This is unique to rodents and NPY is not found in platelets of other mammals. It is not known whether NPY plays a role in platelet aggregation in rat.

NPY may have nonspecific action on the CVS. NPY appears to be able to induce the secretion of histamine from mast cells and subsequently elicit hypotension (Grundemar and Hakanson, 1991; Shen *et al.*, 1991). Interestingly, the mechanism of action is thought to be independent of a classic G-protein coupled receptor, but by a direct action of NPY on the G-protein (Grundemar and Hakanson, 1991; Shen *et al.*, 1991).

The role of NPY in CVS regulation is not completely understood. NPY requires high frequency or burst activity in the postganglionic nerve to be released (Haass *et al.*, 1989; Dahlof *et al.*, 1986; Lundberg *et al.*, 1986; 1989a; Pernow *et al.*, 1989; Schoups *et al.*, 1988; Zukowska-Grojec *et al.*, 1992). This would suggest that NPY may not be released during regular sympathetic control of the CVS. Indeed, it appears that NPY release and subsequent activity only occurs under conditions of hard exercise (Lundberg *et al.*, 1987) or certain forms of stress (Morris *et al.*, 1986; Shen *et al.*, 1991; Takahashi *et al.*, 1988; Zukowska-Grojec and Vaz, 1988). Importantly, it should be noted that increased levels of NPY have been associated with severe cardiovascular stress such as congestive heart failure (Maisel *et al.*, 1989), endotoxic shock (Watson *et al.*, 1988), hypertension (Edvinsson *et al.*, 1991), and open heart surgery (Franco-Cereceda *et al.*, 1990).

Therefore, NPY appears to play an important role in the regulation of the CVS in the periphery, particularly under conditions of physical activity and stress. NPY acts to increase blood pressure and possibly restrict blood flow to various areas of the organism. More importantly, NPY may also play a role in the pathogenicity of certain CVS diseases indicating that the development of drugs directed toward NPY receptors may benefit patients with such diseases.

The effects of NPY on gastro-intestinal function have also been investigated (Cox, 1993). NPY has effects on both secretion and motility of the GI tract. For example, NPY inhibits the secretory effects of PGE$_2$ in the rat jejunum *in vitro* (Saria and Buebler, 1985). NPY or PYY inhibits transport at the basolateral membrane of mucosal sheets (Cox and Cuthbert, 1989). NPY suppresses distension-induced motor activity of the circular muscle of the guinea-pig small intestine (Wiley and Owyang, 1987). Also, peristaltic movements are inhibited and longitudinal smooth muscle of the colon is relaxed (Cox, 1993). Finally, NPY can inhibit both cholinergic and noncholinergic reflexes (Cox, 1993). Therefore, it would appear that NPY has many effects on the functioning of the GI tract.

The previous section has shown that NPY and its receptors are found in high concentration and widely dispersed throughout the entire CNS of mammals (Hendry, 1993). The function of NPY in the CNS is less well understood relative to the periphery. However, some functions are becoming apparent.

As mentioned in the previous section, NPY receptors are found in the dorsal horn of the spinal cord in close apposition to sensory nerve terminals (Bongianni *et al.*, 1990). Intrathecal administration of NPY to the spinal cord produces an antinociceptive effect (Hua *et al.*, 1991). Furthermore, NPY inhibits the release of substance P from sensory neurons in culture (Walker *et al.*, 1988), a transmitter thought to be involved in the relay of nociceptive information. Therefore, NPY may play a role in pain-related information processing at the level of the spinal cord.

NPY appears to contribute to the CNS regulation of the CVS (McAuley *et al.*, 1993). NPY appears to act at the spinal cord, brain stem and hypothalamus. In the spinal cord, injection of NPY intrathecally at levels T_4 or T_{10} causes vasodilation and hypotension (Chen *et al.*, 1990; Westfall *et al.*, 1987). This effect appears to be indirect, through the modulation of adrenergic transmission, as it is inhibited by the alpha-2 receptor antagonist yohimbine, or by prior depletion of NE and EPI with reserpine or 6-hydroxydopamine (Chen *et al.*, 1990). Two areas of the brainstem thought to be important in CNS control of the CVS are the nucleus of the solitary tract (NTS)

(Harfstrand *et al.*, 1987), and the rostral ventral medulla (RVLM) (Ciriello and Caverson, 1986; Ross *et al.*, 1984a,b). Injection of NPY into the NTS causes a pressor response at low doses and a depressor response at higher doses (Carter *et al.*, 1985), This observation is controversial as other investigators found only the depressor response (Tseng *et al.*, 1988). In the RVLM, NPY injection elicits a rapid and transient decrease in heart rate followed by a more sustained increase in blood pressure and heart rate (Tseng *et al.*, 1988). In the area postrema, a circumventricular organ associated with CVS regulation, NPY causes a transient pressor response and increased heart rate followed by hypotension and decreased heart rate (Tseng *et al.*, 1988). Stimulation of the PVN of the hypothalamus causes a pressor response, however, NPY injected into the PVN elicits hypotension (Harland *et al.*, 1988). Therefore, although we are far from understanding the details concerning the role of NPY in the CNS control of the CVS, it is clear that NPY has a function in central cardiovascular regulation at a variety of structures in the CNS.

NPY has been shown to regulate hormone levels and secretion from the hypothalamus and the pituitary (MacDonald and Koenig, 1993). In ovariectomized rats, NPY inhibits the release of luteinizing hormone (LH) from the anterior pituitary with no affect on follicle sitmulating hormone (FSH) (McDonald *et al.*, 1985; Tatemoto *et al.*, 1982). This is thought to be due to NPY inhibition of luteinizing hormone releasing hormone (LHRH) in the hypothalamus (Khorram *et al.*, 1987; McDonald *et al.*, 1989). However, estrogen application changes the effect of NPY to one which causes the release of LHRH and LH (Kalra and Crowley, 1984; Khorram *et al.*, 1987). Furthermore, estrogen causes the release of NPY into the hypophyseal portal sytem and progesterone during the FSH and LH surge increases both NPY and LHRH in the medial basal hypothalamus and preoptic area (Crowely *et al.*, 1989). In addition, NPY can directly cause the release of LH, FSH and growth hormone (GH) from the isolated pituitary and NPY potentiates LHRH-induced LH secretion from from pituitary *in vitro* and *in vivo* (Bauer-Dantoin *et al.*, 1991; Crowley *et al.*, 1987; Sutton *et al.*, 1988). Therefore, NPY appears to modulate the release of hormone at the level of the hypothalamus and directly at the pituitary. NPY's effect depends on the levels of circulating hormones, thus its effect is integrated into the reproductive state of the animal.

NPY may also play a role in the regulation of stress hormones in the periphery by a central mechanism. Introcerebroventricular (ICV) and PVN injections of NPY cause the release of ACTH, corticosterone and aldosterone in male rats (Harfstrand *et al.*, 1987; Wahlestedt *et al.*, 1987). The mechanism of NPY's action is thought to be stimulation of corticotropin-releasing hormone (CRF) release from the PVN because NPY-ir fibers are found in close apposition to CRF neurons. NPY decreases the concentration of CRF in the median eminence which suggests increased release of CRF (Haas and George, 1987), and NPY antibodies block stress-induced release of ACTH (Inui *et al.*, 1990). Therefore, NPY may play a role in controlling hormonal responses to stress.

NPY has also been shown to stimulate activity in supraoptic nuclei (SON) neurons and evoke the release of vasopressin from these same neurons (Day *et al.*, 1985; Willoughby and Blessing, 1987). ICV injection of NPY inhibits the release of GH

(Harfstrand *et al.*, 1987), but also inhibits the release of GH at the pituitary (Chabot *et al.*, 1988; MacDonald *et al.*, 1985). These actions of NPY have not been extensively investigated.

Therefore, it appears that NPY has important functions in regulating physiological levels of hormones. The mechanisms of NPY's actions in this context are not well understood. The effects of NPY are complex acting often at more than one level of hormone release and the modulatory effects of NPY are often influenced by the circulating hormone levels and the physiological state of the animal.

One of the most interesting effects of NPY is its ability to potently and dose-dependently induce eating, even in satiated animals (Clark *et al.*, 1984; Stanley and Leibowitz, 1984; Stanley *et al.*, 1985). NPY can also elicit feeding in other mammals (Miner *et al.*, 1989; Parrot *et al.*, 1986), snakes (Morris and Crews, 1990), and birds (Denbow *et al.*, 1988; Kuenzel *et al.*, 1987). Rat pups as young as 2 days can be induced to eat by NPY, suggesting the effect is not age-dependent (Capuano *et al.*, 1986). The most potent responses to NPY occur in an area called the perifornical area of the hypothalamus (Stanley *et al.*, 1992), which has a high level of PYY binding (Quirion *et al.*, 1990). As mentioned in the section on receptors, the NPY feeding response appears to be mediated by a Y_1-like receptor in that NPY, PYY and $[Pro^{34}]$NPY have potent effects (Kalra *et al.*, 1991a,b; Stanley *et al.*, 1992), however the Y_2 receptor ligand NPY_{2-36} has very potent effects while other C-terminal fragments do not (Jolicoeur *et al.*, 1991; Kalra *et al.*, 1991a,b; Leibowitz and Alexander, 1991; Magdalin *et al.*, 1989; Stanley *et al.*, 1992). This suggests NPY may mediate the feeding response through a novel receptor. It is interesting that NPY-induced feeding is nutrient specific, in that rats will choose carbohydrates over fats or proteins (Morley *et al.*, 1987; Stanley *et al.*, 1985).

NPY may also be associated with obesity. NPY levels increase in normal rats just prior to their nocturnal feeding phase (Jhanwar-Uniyal *et al.*, 1990) and in rats which have been deprived of food for a day (Beck *et al.*, 1990; Calza *et al.*, 1989; Kalra *et al.*, 1991a,b; Sahu *et al.*, 1988). NPY is found in higher concentrations in the extracellular fluid of the PVN and arcuate nucleus of the hypothalamus in spontaneously obese rats (Abe *et al.*, 1991), rats made diabetic with streptozotocin (Sahu *et al.*, 1990; Williams *et al.*, 1989), and in genetically obese Zucker rats (Beck *et al.*, 1990a). Furthermore, NPY levels are increased in the cerebrospinal fluid (CSF) of bulimic patients prevented from binging but not in patients allowed to binge (Berrettini *et al.*, 1988). Therefore, not only may NPY be important in appetite under normal physiological conditions, NPY may also be involved in the pathology of certain eating disorders and eating disorders associated with other diseases.

NPY has other behavioural consequences in the hypothalamus. NPY appears to be important in circadian rhythms as NPY can cause a phase shift in the circadian rhythm when injected into the SCN of the hypothalamus (Albers and Ferris, 1984; Albers *et al.*, 1984). NPY injected into the preoptic area appears to suppress sexual activity (Clark *et al.*, 1986). The effects of NPY on these behaviours are less well understood compared to the feeding response. Therefore, NPY injection into the hypothalamus has several behavioural consequences. However, the cellular actions are less well understood.

NPY has been associated with behavioural disorders such as anxiety and major depression (Heilig *et al.*, 1994). NPY injection into the amygdala produces an anxiolytic effect in rats (Engel *et al.*, 1984; Vogel *et al.*, 1971). This effect is mimicked by Y_1 but not Y_2 agonists (Fuhlendorff *et al.*, 1990; Heilig *et al.*, 1993). Furthermore, antisense oligonucleotide knockout of the Y_1 receptor results in anxiogenesis (Wahlestedt *et al.*, 1993). NPY in the CSF of depressed patients show a negative correlation to their level of anxiety (Heilig *et al.*, 1988; 1989). Taken together, these observations suggests NPY under normal conditions prevents anxiety via the activation of a Y_1 receptor.

NPY has also been correlated with major depression. Decreased levels of NPY are found in the CSF of depression patients compared to normal controls (Widerlov *et al.*, 1988). A reduction in tissue NPY levels is seen in suicide victims and an even larger decrease is seen in tissue from subjects with major depression (de Quidt and Emson, 1986). Antidepressant treatments such as electroconvulsive therapy (Wahlestedt *et al.*, 1990a) and drug therapy (Heilig *et al.*, 1988a) increase the levels of NPY in the cortex and hypothalamus of rats. Therefore, NPY may be involved in controlling the emotional state of an animal and may be involved in some of the pathologies associated with certain behaviours.

Finally, NPY may modulate memory in the mouse (Flood *et al.*, 1987). When NPY is injected ICV, directly into the rostral hippocampus or into the septum, memory retention is improved (Flood *et al.*, 1987; 1989). In contrast, NPY injected into the caudal hippocampus or the amygdala inhibits memory retention (Flood *et al.*, 1989). These effects could be mimicked by Y_2 agonists and NPY antibodies had opposing effects

(Flood and Morley, 1989; Flood *et al.*, 1989). Thus NPY may play a role in modulating memory in mammals.

Therefore not only is NPY and its receptors widely dispersed throughout the periphery and CNS, but NPY also appears to have a plethora of physiological functions. In addition to having physiological functions, it appears NPY may be associated with disease states in the periphery and the CNS. This may make NPY receptors interesting targets for a variety of pharmacological therapies (Wahlestedt and Reis, 1993). However, the cellular mechanisms by which NPY exerts these physiological actions are less well understood. This is particularly the case for the CNS, where it has been shown in this and the previous section that NPY and its receptors are found in great abundance and have several physiological and behavioural effects.

Cellular Mechanisms of NPY's Actions in the Periphery and CNS

As mentioned in previous sections, NPY has both presynaptic and postsynaptic activities at the sympathetic neuroeffector junction on vascular smooth muscle. Postsynaptically, NPY has a direct and indirect effect (potentiating adrenergic vasoconstriction) causing vasoconstriction of smooth muscle. These effects can be blocked by voltage-dependent calcium channel (VDCC) blockers (Cheung, 1991; Pernow *et al.*, 1986; Small *et al.*, 1992), suggesting the involvement of VDCCs. Subsequently, L-type VDCCs have been shown to be potentiated by NPY in smooth muscle cells (Xiong *et al.*, 1993). Furthermore, NPY has recently been shown to inhibit calcium-activated potassium channels (K_{Ca}) in smooth muscle cells as well (Xiong and Cheung, 1994). The activation of K_{Ca} may act as a negative feedback mechanism to limit the amount of calcium entering the smooth muscle cell through VDCCs (Brayen and Nelson, 1992). If so, NPY may potentiate vasoconstriction via two interacting mechanisms, a direct potentiation of L-type VDCCs and an inhibition of K_{Ca}.

As previously mentioned, NPY inhibits the release of substance P from cultured sensory neurons from dorsal root ganglia (DRG) (Walker *et al.*, 1988) and acetylcholine (Ach) from nodose ganglia cells from rat (Wiley *et al.*, 1990). Furthermore, NPY terminals are seen to come in close apposition to sensory fibers in the spinal cord (Bongianni *et al.*, 1990). In DRG cells, NPY inhibits action potential (AP) induced increases in intracellular calcium (Bleakman *et al.*, 1991) and VDCCs (Bleakman *et al.*, 1991; Thayer and Miller, 1990; Walker *et al.*, 1988). This action can be mimicked by C-terminal fragments, suggesting the process is mediated by a Y_2 receptor (Bleakman *et al.*, 1991). This inhibition is blocked by PTX but the effect of NPY can be reestablished by intracellular perfusion with the α subunit of the G_o-protein (Ewald *et al.*, 1989) suggesting NPY is coupled to the G_o-protein subtype. NPY's actions in nodose ganglia cells are more complicated. NPY appears to inhibit N-type VDCCs in nodose ganglia cells activation of Y_2 receptors, but in a small population of cells, the same VDCCs can be potentiated by the activation of a Y_1 receptor (Wiley *et al.*, 1993). The functional consequences of the finding that NPY can modulate VDCCs in the cell bodies of these sensory neurons is unknown. However, given that NPY inhibits the release of transmitter from the terminals of these sensory neurons, it is tempting to extrapolate these findings to the terminal where NPY may inhibit transmitter release by inhibiting Ca^{2+} influx as occurs in sympathetic neurons.

The cellular mechanism by which NPY's inhibits transmitter release from sympathetic nerve terminals has also been investigated in some detail. VDCC's in the somata of vertebrate sympathetic neurons are inhibited by NPY (Schofield and Ikeda, 1988; Plummer *et al.*, 1991; Foucart *et al.*, 1993; Toth *et al.*, 1993). In frog sympathetic C cells, NPY activates an inwardly rectifying K^+ channel (Zidichouski *et al.*, 1990) as well as inhibiting VDCCs (Schofield and Ikeda, 1988). However, most of these studies measured voltage-clamped currents from cell somas, which may not be an accurate reflection of activity at the nerve terminal where NPY inhibits the release of NE (Lundberg *et al.*, 1982; 1989). Toth and colleagues (1993) have used cocultures of sympathetic neurons and atrial myocytes to more closely examine the actions at the terminals. They have shown that NPY inhibits transmitter release from terminals in culture by inhibiting Ca^{2+} influx. The effect of NPY is occluded by ω-CTX-GVIA. These data suggest that NPY is inhibiting transmitter release from sympathetic nerve terminals by inhibiting Ca^{2+} influx through N-type VDCCs in the terminal (Toth *et al.*, 1993).

As discussed previously, NPY has several physiological actions in the GI system including inhibition of secretion, GI motility and cholinergic and noncholinergic reflexes (Cox, 1993). However, the cellular actions of NPY have not been investigated in great depth. NPY has been shown to inhibit N-type VDCCs in PTX-sensitive and membrane delimited manner in cultured myenteric neurons of the rat (Hirning *et al.*, 1990). The function of this action is unknown.

In the CNS, the cellular actions of NPY have been investigated in the locus coeruleus (LC), the dorsal raphe and the hippocampus (Colmers and Bleakman, 1994).

The cellular actions of NPY in the brain stem nuclei will be described first.

In the LC, NPY has been shown to reduce the frequency of APs in LC neurons (Illes and Regenold, 1990). The effect was shown to be indirect by potentiating the postsynaptic hyperpolarizing action of α_2 receptor activation (Illes and Regenold, 1990). Interestingly, NPY does not affect the action of mu opioid receptors which activate the same K^+ channel in the identical cell type (North and Williams, 1985) suggesting that NPY is interacting with the α_2 receptor or its coupling mechanism. Although NPY also potentiates adrenoceptor activation on vascular smooth muscle (Wahlestedt and Reis, 1993), the effects appear to be different. NPY potentiates the α_2 receptor in the LC (Illes and Regenold, 1990) and the α_1 receptor in smooth muscle (Wahlestedt and Reis, 1993). Furthermore, NPY acts via a Y_2 receptor in the LC (Illes *et al.*, 1993) and a Y_1 receptor in vascular smooth muscle (Wahlestedt and Reis, 1993). The cellular actions of NPY in the LC may be part of the mechanism by which NPY is involved in anxiolysis (Heilig, 1993), since a reduction in the firing rate of LC neurons results in anxiolysis (Redmond and Huang, 1979).

The dorsal raphe nucleus is the source of most of the serotonergic input to the forebrain. The nucleus consists of largely serotonergic neurons which have glutamate and GABA receptors that mediate fast excitatory and inhibitory synaptic potentials. Furthermore, raphe neurons also have 5-HT_{1A} receptors which act as autoreceptors which hyperpolarize raphe neurons to limit the amount of firing (Williams *et al.*, 1988; Yoshimura and Higashi, 1985) and NE inputs from outside the nucleus which activate α_1 receptors causing the raphe neurons to depolarise and fire tonically (Williams *et al.*, 1988). NPY is also found in the dorsal raphe nucleus and has been shown to presynaptically inhibit NE and 5-HT release onto serotonergic neurons of the raphe (Kombian and Colmers, 1992). These actions can be mimicked by an agonist profile consistent with the activation of a Y_2 receptor (Kombian and Colmers, 1992). NPY had no affect on the passive electrical properties of raphe neurons and had no affect on glutamatergic or GABAergic transmission in the raphe. Although the consequences of these cellular actions are not completely understood, these authors suggest that NPY, by inhibiting NE release, will reduce the tonic firing of raphe neurons, whereas its inhibition of 5-HT release would allow for more prolonged activity (by suppressing the activity-dependent autoinhibition) when these neurons were excited (Kombian and Colmers, 1992). Thus the authors compare their observations to the model of "quiet readiness" described for NE action in the cortex (Livingstone and Hubel, 1981). In this scenario, upon release of NPY, the raphe neurons activity would be suppressed through the inhibition of tonic NE input. However, with other excitatory input, such as glutamatergic, the neurons would fire with a more prolonged activity, since the autoreceptor activation has been suppressed (Kombian and Colmers, 1992).

The Mammalian Hippocampal Formation

The hippocampus is probably the most intensively studied area of the mammalian brain in terms of neuromodulation (Nicoll *et al.*, 1990). In addition, the cellular actions of NPY in the CNS have received the most attention in the hippocampus (Colmers and Bleakman, 1994). For these and other reasons, NPY's cellular actions in the hippocampus is the focus of this thesis.

Neuromodulation has been intensively studied in the hippocampus because of its simple anatomical structure and the behavioral functions of the hippocampus. In the early 1970's, the hippocampus was postulated to process information through a simple, three-synapse relay (Andersen *et al.*, 1971). The circuit involved the collection of multimodal sensory information from various sensory associational areas of the neocortex by the entorhinal cortex (EC) (Amaral, 1993; Amaral and Witter, 1989; Boenijinga and Lopes da Silva, 1988; Eichenbaum *et al.*, 1992; Room and Groenewegen, 1986; Van Hoesen *et al.*, 1972; Yellin and Jerison, 1982). The EC then relays the information through the hippocampus via a trisynaptic network which involves in sequence: the EC projecting to the dentate granule cells (DGC) of the dentate gyrus via the perforant path, the DGCs relay the information onto the CA3 pyramidal neurons via the mossy fibers, the CA3 pyramidal cells then project to the CA1 pyramidal cells via the Schaffer collaterals, and the CA1 pyramidal cells then pass the processed information out through the subiculum (Andersen et al., 1971). Furthermore, the entire circuit was thought to be contained in discrete lamina, transverse to the longitudinal or the septo-temporal axis of the hippocampus (Andersen *et al*,. 1971). This permitted sectioning of the hippocampus into 400 to 500 μm slices along the transverse plane while maintaining intact the internal circuitry of the hippocampus. The hippocampal slice preparation spawned a great deal of research on the modulation of cell properties and synapses of the hippocampus (Nicoll *et al.*, 1990). However, subsequent anatomical research has shown that the circuitry of the hippocampus is not this simple (Amaral, 1993).

The neural circuitry of the hippocampus is neither entirely lamellar, nor trisynaptic (Amaral and Witter, 1991; Amaral, 1993). The perforant path from the EC is a good example of how the circuitry diverges from these two criteria. The perforant path from a small, discrete area of the EC collateralizes extensively along the entire longitudinal axis of the hippocampus and is therefore not laminar (Hjorth-Simonsen and Jeune, 1972; Nafstad, 1967; Ruth *et al.*, 1988; Steward, 1976; Struble *et al.*, 1978; Witter and Groenewegen, 1984; Witter *et al.*, 1988; 1989). Furthermore, the perforant path does not only project to DGCs of the dentate gyrus, the first synapse of the "trisynaptic" network, but also projects to CA3 and CA1 pyramidal neurons and subicular neurons (Steward and Scoville, 1976; Witter and Amaral, 1991; Witter *et al.*, 1988). The hilar neurons of the dentate gyrus, which are thought to feedback and control DG output, do not project within their own transverse lamella but instead project preferentially within the ipsilateral DG and to the contralateral DG, outside their own lamella (Laurberg, 1979; Laurberg and Sorensen, 1981; Swanson *et al.*, 1978). The CA3 associational projection to other CA3 neurons, and through the Schaffer collaterals to CA1 pyramidal cells, also diverges substantially from its transverse lamella (Amaral and Witter, 1989; Ishizuka *et al.*, 1990). Inhibitory interneurons of all areas of the hippocampus produce axons which collateralize massively both within and outside of the transverse axis (Buszaki, 1984; Struble *et al.*,1978; Swanson *et al.*, 1987). In fact, the only truly laminar projection within the trisynaptic circuit which restricts its synaptic interaction to the transverse plane of its origin is the mossy fiber projection from DGC's to CA3 pyramidal neurons (Blakstadt *et al.*, 1970; Gaarskjaer, 1978; 1986; Swanson *et al.*, 1978).

Therefore, a great deal of circuitry of the hippocampus is not preserved in transverse slices of the hippocampus and as such due to the elimination of the functional circuitry of the hippocampus cannot be determined from such slices as a lot of longitudinal interactions have been eliminated. However, much useful information can be obtained from transverse hippocampal slices, as intact and cut axons can still be stimulated and postsynaptic responses recorded. Synaptic responses of cells can be recorded extracellularly or intracellularly, active and passive neuronal properties can be stably recorded and the effects of drugs on any or all of these responses can be rigorously examined in hippocampal slices. However, a full understanding of the hippocampal circuitry *in vivo* cannot be obtained solely from the hippocampal slice.

The hippocampus has also generated interest due to its putative role in higher-order functions in the CNS. As mentioned above, the hippocampus receives many projections from higher order areas involved in multimodal sensory information processing (Lopes da Silva *et al.*, 1990). The hippocampus has been implicated in memory formation for several decades now (Eichenbaum *et al.*, 1992). However, the hippocampus does not appear to permanently store memories and is not involved in the formation of all types of memory (Cohen, 1984). The hippocampus appears to be involved in the formation of declarative memory, i.e. memory where relationships are drawn from multiple items and events which then can be used in a flexible manner in novel situations (Eichenbaum *et al.*, 1992). In contrast, the hippocampus is not needed for the formation of procedural memory, in which a skill is involved and the memory is only used during the repetition of the learned event (Eichenbaum *et al.*, 1992). The hippocampus has also been thought to belong to a spatial mapping system (O'Keefe and Conway, 1978) and place learning (Morris *et al.*, 1982; Eichenbaum *et al.*, 1990). Principal cells of the hippocampus fire when the animal occupies a particular spatial area in a well-defined environment (O'Keefe, 1976; 1979). These neurons were called place cells (O'Keefe and Nadel, 1978). However, principal cells of the hippocampus have been shown to fire in association with other tasks such as odor discrimination (Eichenbaum *et al.*, 1992) and conditioned eyeblinks and jaw movements in rabbits (Berger *et al.*, 1986). Furthermore, a single cell can have both a place cell function and an odor discrimination function (Eichenbaum *et al.*, 1992), and place cells change unpredictably when the animal is placed in a different environment (Muller and Kubie, 1987). Therefore, the functional representation in the hippocampus and within individual cells of the hippocampus changes with the task at hand.

The hippocampus also displays behaviorally related electrical activity. The best described is theta rhythm, which has been associated with exploratory behaviour. For example theta rhythm has been phase locked to sniffing rhythm in rats (Kamisruk, 1970; Eichenbaum *et al.*, 1992). Theta rhythm is a rhythmic oscillation in a population of neurons of the hippocampus. The oscillations can be recorded from the population of neurons, unit recordings of single cells or intracellular membrane recordings from individual neurons (Lopez da Silva *et al.*, 1990). There appears to be two types of theta, varying in frequency from 4 to 12 Hz. Type 1 appears to be associated with voluntary motor activity such as walking and swimming, whereas type 2 is associated with consumatory behaviours such as eating, drinking and grooming (Leung *et al.*, 1982).

Type 2 is not as prevalent as type 1 theta. Theta appears to involve acetylcholine input from the septum (Lopez da Silva *et al.*, 1990; Traub and Miles, 1991). However, the cellular mechanism, which has been intensively investigated, is still controversial (Traub and Miles, 1991; Lopez da Silva *et al.*, 1990). Some investigators favour theta rhythmic synchronization of principal cells through synchronous EPSPs in the dendrites (Fujita and Sato, 1964; Nunez *et al.*, 1987; 1990; Konopacki *et al.*, 1992), while others favour the synchronization of interneurons which then in turn synchronize the principal cells of the hippocampus via IPSPs (Artemenko, 1972; Leung and Yim, 1986; Fox, 1989; Soltesz and Deschenes, 1993; Ylinen *et al.*, 1995). The latter hypothesis is consistent with the septum as the rhythm generator (Gray, 1971; Winson, 1978). GABA inputs from the septum selectively innervate interneurons of the hippocampus (Freund and Antal, 1988) to produce rhythmic release of GABA onto hippocampal interneurons (Ylinen *et al.*, 1995). The hippocampal interneurons in turn inhibit principal neurons of the hippocampus at the same rhythm generated in the septum (Ylinen *et al.*, 1995). Interestingly, burst theta rhythm is an efficient means of inducing long term potentiation (LTP) (O'Keefe and Nadel, 1978), a cellular model for learning and memory based on modification of synaptic efficacy (Bliss and Collingridge, 1993). Theta rhythm may act to increase the activation of NMDA receptors, which is required for some types of LTP, and theta may suppress inhibitory synaptic transmission (Larson and Lynch, 1988), which would aid in the generation of LTP. Other behaviourally relevant rhythms have also been observed in the hippocampus. These include beta or gamma waves (20-100 Hz) seen in active, freely-moving rats or rats in REM sleep and interspersed with theta (Boudreau, 1966; Stumpf, 1965), EEG spikes (less than 70 ms duration) and sharp waves (70-200 ms duration) which are the result of synchronized hippocampal neurons bursting which has been associated with involuntary behaviours such as feeding, drinking and grooming (Buzsaki, 1986; Buzsaki *et al.*, 1983; Suzuki and Smith, 1985, 1987, 1988a-d). However, the cellular mechanisms of these rhythms are poorly understood.

The hippocampus is also a site for the investigation of pathologies. Many epileptic patients have seizures which originate at a focal point in the brain which subsequently spread to other areas. Many of these foci are in the medial temporal lobe of the cortex, quite often the hippocampus (Gastaut, 1970). Thus a great deal of effort has been made in an attempt to understand excitability in the hippocampus with the expectation that such investigations would lead to a subsequent understanding of the abnormalities which cause overexcitability and epilepsy.

Therefore, the hippocampus appears to be an area of the brain which is of outstanding interest in CNS function. It has an important function in declarative memory, has obvious behavioural associations in its electrical activity and may be a major site of certain brain pathologies such as amnesia and epilepsy. In the next section, the cellular actions of NPY in the hippocampus are described.

Cellular Actions of NPY in the Mammalian Hippocampal Formation

As mentioned above, NPY and its receptors are found in large concentrations throughout the hippocampus (Hendry, 1993). NPY is localized to interneurons in the hippocampus itself, particularly in cells of the dentate hilus but it is also found in cells of the strata pyramidalia, radiatum and oriens (Hendry, 1993). Interestingly, the DGCs, which do not normally synthesize NPY, can be induced to express NPY and its mRNA following the induction of limbic seizures in the rat (Colmers *et al.*, 1992; Gall *et al.*, 1989; Marksteiner *et al.*, 1989; Martel *et al.*, 1990).The receptors for NPY are found in the stratum radiatum and stratum oriens of the CA subfields of the hippocampus proper and in the inner one third of the molecular layer of the dentate gyrus (Kohler *et al.*, 1986; 1987; Lynch *et al.*, 1989; Martel *et al.*, 1986; Schwerdtfeger and Buhl, 1986). These are areas where excitatory synapses are made onto CA1 and CA3 pyramidal neurons and where hilar neurons input onto dentate granule cells. The receptors for NPY in the CA subfields appear to be presynaptic since selective destruction of pyramidal cells with quinolinic acid does not eliminate NPY binding (Lynch *et al.*, 1989). Furthermore, the receptor subtypes in the hippocampus proper appear to be Y_2, which have been shown to inhibit transmitter release in the periphery (Toth *et al.*, 1993). Interestingly, however, the receptor subtype in the dentate gyrus appears to be Y_1, the receptor subtype which is mainly associated with postsynaptic activity (Wahlestedt and Reis, 1993).

From the anatomical data above, a reasonable hypothesis would be that NPY may modulate excitatory synaptic transmission in the CA subfields of the hippocampus proper and modulate synaptic transmission of hilar cells of the dentate gyrus or the postsynaptic activity of dentate cells.

Indeed, in the CA subfield of the hippocampus, NPY appears to presynaptically inhibit excitatory synaptic transmission. This was first demonstrated and most extensively investigated in the CA1 region of the rat hippocampal slice. There, NPY has been shown to inhibit extracellularly evoked excitatory synaptic transmission without affecting the passive electrical properties of the CA1 pyramidal neurons (ie. their resting potential or input resistance) (Colmers *et al.*, 1987; 1988). NPY did not alter iontophoretic applications of glutamate to the postsynaptic CA1 pyramidal cell (Colmers *et al.*, 1988). The effect of NPY was also resistant to the K^+ channel blocker, 4-aminopyridine (Colmers *et al.*, 1988; Klapstein and Colmers, 1992). This inhibition could be mimicked by PYY and C-terminal fragments (Colmers *et al.*, 1991). Furthermore, NPY and Y_2-selective agonists inhibited the K^+-evoked release of glutamate from rat hippocampal slices (Greber *et al.*, 1994). However, NPY does not appear to affect inhibitory synaptic transmission onto CA1 pyramidal neurons (Klapstein and Colmers, 1993). Monosynaptic inhibitory postsynaptic potentials (IPSP), recorded in pharmacological isolation, are unaffected by NPY in both CA1 and CA3 (Klapstein and Colmers, 1993). Interestingly, the recurrent IPSPs are also unaffected by NPY (Klapstein and Colmers, 1993). However, feedforward inhibitory synaptic transmission appears to be indirectly inhibited by NPY, probably via its inhibition of upstream feedforward excitatory synaptic transmission. Taken together, NPY appears to selectively inhibit excitatory synaptic transmission in CA1 of the rat hippocampus by a presynaptic mechanism.

Similar findings have been made in CA3 pyramidal cells in the rat hippocampal slice. Again, NPY inhibits evoked-EPSPs in CA3 pyramidal cells without affecting the passive electrical properties of the postsynaptic cell (Klapstein and Colmers, 1993). The receptor subtype and the site of action were presumed to be similar to NPY's action in CA1 but has not been directly investigated (Klapstein and Colmers, 1993). Inhibitory synaptic transmission in this region was not directly affected (Klapstein and Colmers, 1993). It is interesting that, *in vivo*, NPY has been shown to potentiate NMDA-evoked activity in the CA3 region of the rat via the interaction with a sigma receptor (Monnet *et al.*, 1990, 1992a,b). However, such observations were not made in the studies of intracellular recordings from CA3 pyramidal cells of the rat hippocampal slice (Klapstein and Colmers, 1993). This may be the result of poor activation of NMDA receptors from evoked EPSPs owing to the NMDA receptor's voltage-dependence (Mayer *et al.*, 1984; Nowak *et al.*, 1984) or due to the overriding influence of the inhibition of excitatory synaptic transmission produced by NPY. Clearly, more direct experiments are required to adequately demonstrate putative ability of NPY to potentiate NMDA receptors. At this point we may summarize that NPY appears to inhibit excitatory synaptic transmission in CA3 of the rat hippocampus as it does in CA1.

In the dentate gyrus, NPY does not appear to have an effect on either the excitatory or inhibitory synaptic transmission onto DGCs (Klapstein and Colmers, 1993). Furthermore, NPY does not affect the passive electrical properties of DGCs or the frequency of evoked APs in DGCs (Klapstein and Colmers, 1993). One study has suggested that NPY directly depolarizes DGC neurons (Brooks *et al.*, 1987), however no change in resting potential to NPY application was observed by other investigators (Klapstein and Colmers, 1993). Brooks *et al.*, (1987) applied NPY by pressure ejection into the hippocampal slice, and measured the postsynaptic responses, a procedure prone to mechanical artefact (Brooks *et al.*, 1987). In contrast, bath applied the NPY in the slice, produced no effect (Klapstein and Colmers, 1993). Therefore, an electrophysiological function for NPY receptors in the DGC remains to be determined.

Hypotheses

The studies of this thesis were carried out to gain a better understanding of the cellular actions of NPY in the rat hippocampus. The thesis has three parts. First, a study was undertaken to examine if NPY can potentiate the NMDA receptor on CA3 pyramidal neurons by simultaneously examining NPY's effect on the response to currents caused by iontophoretically applied NMDA and synaptic stimulation of CA3 pyramidal cells of rat hippocampal slices. Second, the site (presynaptic vs. postsynaptic) and mechanism of action of NPY on excitatory synaptic transmission onto CA3 pyramidal cells of the rat hippocampus was more closely examined by determining the receptor subtype and measuring the effect of NPY on spontaneous (sEPSC) and miniature excitatory synaptic currents (mEPSC). It is expected, according to the quantal hypothesis of Fatt and Katz, 1952, that NPY will act presynaptically through a Y_2 receptor as demonstrated by NPY and Y_2 agonists affecting the frequency of sEPSCs without affecting their amplitudes. Lastly, since NPY appears to act through Y_1 receptors in the DGC and Y_1 receptors have been linked to potentiation of VDCCs and release of intracellular Ca^{2+}, we hypothesize that NPY may increase the $[Ca^{2+}]_i$ of DGCs or potentiate VDCCs of DGCs of the rat hippocampus. This has been tested by measuring $[Ca^{2+}]_i$, Ca^{2+} influx and calcium currents in DGCs of rat hippocampal slices and acutely dissociated DGCs from the rat hippocampus.

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Chapter 2

Neuropeptide Y does not alter NMDA conductances in CA3 pyramidal neurons: a slice-patch study

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Neuropeptide Y (NPY) has been shown to inhibit excitatory synaptic transmission in the rat hippocampus by a presynaptic mechanism [5,7,8]. However, a recent study suggests that ligands which bind to σ or phencyclidine (PCP) receptors can be displaced specifically and with high affinity by NPY [15]. Furthermore, another study reported that NPY selectively potentiated extracellularly recorded excitatory responses elicited by microiontophoresis of NMDA onto rat hippocampal CA3 pyramidal neurons *in vivo* but not those caused by quisqualate or kainate [13]. NPY has been shown to modulate the activity of voltage- and ligand-gated ion channels, such as voltage-dependent Ca^{2+} channels [20], a nicotinic acetylcholine receptor [14], and K^+ channels activated by α_2 adrenoceptors [11]. As σ and PCP ligands can modulate responses of the NMDA receptor to agonists [19], it was hypothesized that an NPY action at a σ or PCP binding site was responsible for the observed potentiation of NMDA responses [13]. We have tested this hypothesis by examining the action of NPY on membrane ion currents elicited by iontophoretic application of NMDA to CA3 pyramidal neurons in the *in vitro* hippocampal slice using tight seal, whole-cell voltage clamp ("slice-patch") recordings [3,12,17].

Hippocampal slices (450-500 μm thick) were prepared from male Sprague-Dawley rats (100-200 g) by vibratome sectioning, and maintained in artificial cerebrospinal fluid (aCSF), containing (in mM): NaCl 126, KCl 2.5, $MgCl_2 6H_2O$ 2, $CaCl_2 6H_2O$ 2, $NaH_2PO_4 2H_2O$ 1.25, $NaHCO_3$ 26, glucose 10, which was continuously bubbled with 95% O_2/5% CO_2 (pH 7.35). A single slice was transferred to a small (300 μl) recording chamber [21,7], where it was submerged in aCSF, and supported on a nylon mesh. The slice was continually perfused at 2-3 ml min^{-1} with bubbled aCSF (34 ± 0.5 °C). Patch clamp type pipettes (3-7 MΩ) were filled with either Cs$^+$ electrolyte, containing (in mM): Cs$^+$ gluconate 135, HEPES 10, $MgCl_2 6H_2O$ 2, or K$^+$ electrolyte, containing (in mM): K$^+$ gluconate 145, $MgCl_2 6H_2O$ 2, HEPES 5, EGTA 1.1, $CaCl_2 6H_2O$ 0.1, NaATP 5, NaGTP 0.3. Both electrolytes were buffered to 7.2 and their osmolarity adjusted to 270-280 mOsm. Tight seal, whole cell recordings [11] were obtained from CA3 pyramidal neurons in midtemporal slices using the slice-patch technique [3,12,17]. Seal resistances prior to rupturing the patch were typically $>$ 5 GΩ. The electrode was connected to the headstage of an Axoclamp 2A amplifier, used in the continuous single electrode voltage clamp mode. Recordings were stable, lasting routinely two to three hours, with little change in holding current. Once a stable voltage clamp recording was established, a microelectrode containing NMDA (50 mM, pH 8.0, 100 MΩ), connected to the second channel of the Axoclamp (x1 headstage), was positioned near the proximal dendrites of the cell so as to elicit a response to brief (10-100 msec, -30 to -190 nA, 0.05 Hz) iontophoretic ejections of NMDA. Iontophoretic current duration and amplitude were adjusted in all experiments to produce inward current responses of approximately 200 - 400 pA. Criteria for an acceptable iontophoretic response included rapid onset, a duration which outlasted passage of iontophoretic current, a graded onset and decay, and abolition of the response when current polarity was reversed. In some experiments, a bipolar tungsten stimulating electrode was placed on the mossy fiber pathway in the hilus of dentate and stimuli (10-25V, 50-200 μs) from a stimulus isolator (AMPI) were used to elicit excitatory postsynaptic currents (EPSC's) in the cell. In these experiments, synaptic stimuli were alternated with NMDA iontophoretic pulses during the course of the experiment.

Drugs were applied via the bath. In some experiments, picrotoxin (PcTX, 100 μM) was applied to block $GABA_A$ responses. In other experiments tetrodotoxin (TTX) was applied to block action potentials. Once stable control responses had been obtained to NMDA application and to synaptic stimuli (when applied), NPY (Richelieu Biotechnologies, Quebec) was applied at 1 μM via the bath. Data from experiments where the iontophoretic electrode had moved (as shown by a change in the time of onset of the iontophoretic response) were rejected. Similarly, data were rejected from experiments in which synaptic responses failed to show significant recovery from NPY application.

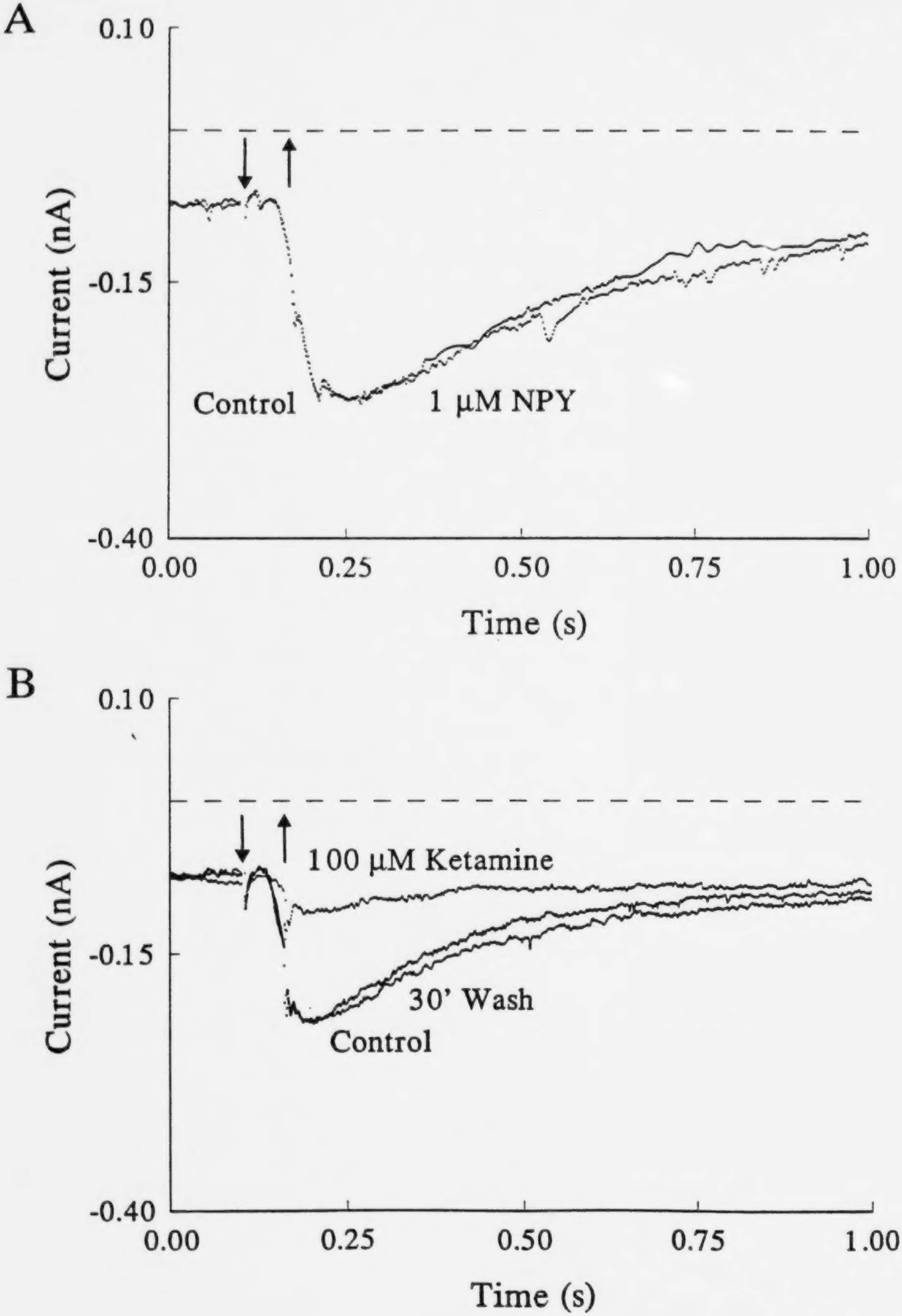
Data were acquired, stored and analyzed with a computer (Northgate 486) using pClamp software (Axon). Digitization rates were optimized to acquire clear EPSC or NMDA response. Data from portions of the experiment were also recorded on PCM coded (44 kHz) videotape for subsequent analysis. Neurons were used as their own controls for drug effects; controls taken immediately before each drug application were used for comparison. Statistical comparisons of drug effect on a given cell were made using Student's paired t-test. Drug effects were calculated as a percentage of peak control responses; these values were used to compare drug actions on the population of cells studied, using a Student's t-test for two means. Differences were considered significant at the $P < 0.05$ level.

Figure 2-1A shows the membrane response of a CA3 pyramidal neuron, held at -

7 mV in TTX, to the iontophoretic application of NMDA. Bath application of 1 μM NPY caused no change in the response to NMDA over a 20 minute period during and after the application of NPY [10]. In this neuron, however, subsequent bath application of 100 μM ketamine caused a pronounced, reversible reduction in NMDA response (Fig. 2-1B); similar results were obtained in two further cells. The mean peak current evoked by NMDA in these neurons in the presence of NPY was 98.50 ± 0.69 % of control values ($n = 3$, $P > 0.1$). Resting membrane current was also not affected by NPY (Fig. 2-1).

Since other investigators have measured the reversal potential of the NMDA response to be -10 to -7 mV [10], one may expect to observe no NMDA current at V_h = -7 mV. However, in agreement with neuronal models [22], the ability to clamp the membrane voltage of the dendrites from the soma is likely to be limited so that although the soma is being clamped at -7 mV, the dendritic membrane where the NMDA responses are being produced is likely to be significantly more negative than -7 mV [22]. Additionally, we measured currents from pyramidal cell of area CA3 as opposed to area CA1, and our intracellular Na^+ concentrations were lower than in Hestrin et al., [10], which would shift the reversal potential of the NMDA current to a more positive voltage, as the NMDA current is carried partially by Na^+ [1]. Because NMDA is a highly selective agonist, because the response elicited by NMDA iontophoresis here is sensitive to ketamine [1], and because of the above space clamp considerations, the response observed is purely an NMDA response although the reversal potential differs somewhat from other studies [10]. This difference does not affect the conclusions of the present study.

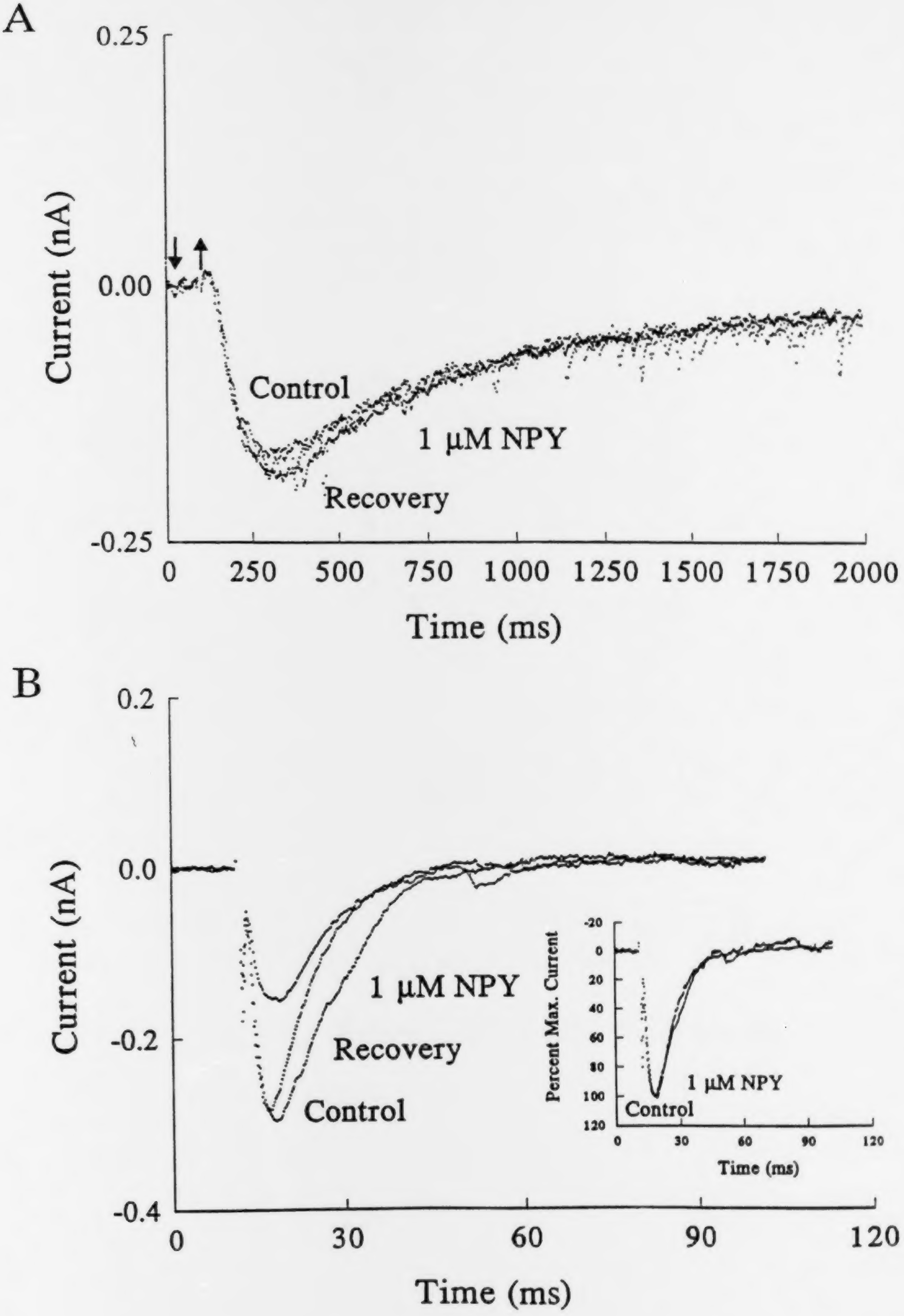
Figure 2-1. Whole cell voltage clamp recording (V_h = -7 mV) of iontophoretically induced NMDA currents in a CA3 pyramidal cell of a hippocampal slice from the rat. Whole-cell pipette contained Cs^+ gluconate; the cell was depolarized to + 20 mV, then gradually returned to - 7 mV, to inactivate voltage-dependent inward currents, prior to the start of the experiment. NMDA pipette was placed near proximal dendrites in stratum radiatum. Current pulses (100 ms, -190 nA) were used to eject NMDA. Arrows indicate on and off current artifact from iontophoresis. A. NMDA currents before treatment and after treatment with 1 μM NPY. B. 60 minutes following washout of NPY, NMDA currents are inhibited by 100 μM ketamine, and return to near control values upon washout.


A
0.10
-0.15
-0.40
Current (nA)
0.00
0.25
0.50
0.75
1.00
Time (s)
Control
1 µM NPY
B
0.10
-0.15
-0.40
Current (nA)
100 µM Ketamine
30' Wash
Control
0.00
0.25
0.50
0.75
1.00
Time (s)

Because NPY diffuses slowly through slice tissue [7,8], we sought to ensure that NPY reached the cell by monitoring its action on an independent response. Mossy fiber EPSC's were therefore evoked in PcTX, to block IPSC's caused by $GABA_A$ receptors. For these experiments, the membrane potential had to be held more negative than in the previous experiments to enable us to evoke a clearly measurable mossy fiber EPSC without having to employ very strong synaptic stimuli. Larger stimulations evoked secondary bursts in the PcTX treated slice. The pipette contained K^+ gluconate, to enable us to observe any possible modulatory effect of NPY on resting K^+ conductances. Fig. 2-2A shows the iontophoretic response to NMDA in one such CA3 pyramidal cell. NPY ($1\mu M$) had no significant effect on the NMDA response, but the EPSC evoked by mossy fiber stimulation was attenuated by about 50 % (Fig. 2-2B). The kinetics of the EPSC are not altered by NPY (Inset, Fig. 2-2B), consistent with a presynaptic site of action. The EPSC recovers upon washout of NPY, while the NMDA response remains unaffected (Fig. 2-2). On average, NPY reduced the mossy fiber EPSC to 53.90 ± 3.74 % of control ($n=5$, $P<0.001$) while in the same cells the NMDA response remained 99.34 ± 1.92 % of control ($n=5$, $P>0.25$). In this and other CA3 neurons tested, NPY caused no change in baseline holding current (Fig. 2-2), consistent with previous observations in hippocampus [4,7,8]. We also often observed spontaneously-occurring miniature EPSC's in the CA3 neurons under these conditions, as has been reported in dentate granule cells [17]. Interestingly, NPY caused a reversible reduction in the frequency of these miniature EPSC's (not illustrated).

The present data do not support the hypothesis that NPY can potentiate the

Figure 2-2. Whole cell voltage clamp recording (V_h = -60 mV) of a CA3 pyramidal cell treated with 100 μM PcTX from a hippocampal slice from a rat. Pipette contained K^+ gluconate. A. NMDA currents elicited by iontophoresis of NMDA onto the proximal apical dendritic field of the cell. Iontophoretic pulses were 100ms, -190 nA. Arrows indicate on and off artifacts of iontophoretic current. Application of NPY (1 μM) did not affect the NMDA current. B. In the same experiment, the EPSC, evoked in the cell by a 24 V, 100 μs stimulus to the mossy fibers was significantly reduced by NPY in a reversible fashion. Inset: NPY response in B was scaled to the same size as control, illustrating that NPY does not alter the kinetics of the EPSC.


A
0.25
0.00
-0.25
Current (nA)
Control
1 μM NPY
Recovery
0
250
500
750
1000
1250
1500
1750
2000
Time (ms)
B
0.2
0.0
-0.2
-0.4
Current (nA)
1 μM NPY
Recovery
Control
0
30
60
90
120
Time (ms)
-20
0
20
40
60
80
100
120
Percent Max. Current
1 μM NPY
Control
0
30
60
90
120
Time (ms)

excitatory response mediated by NMDA in CA3 neurons. No significant change, either a reduction or an increase, was seen in the NMDA response either during the peak response to NPY (measured by the inhibition of EPSC's), or later. The data are consistent with earlier results which demonstrated a presynaptic inhibition of excitatory synaptic potentials by NPY [4,7]. The results suggets that it is unlikely that the observed changes of the NMDA iontophoretic responses in CA3 neurons *in vivo* are due to actions by NPY at a site on the NMDA receptor [13]. In this respect, a recent report suggests that the reported displacement of σ and PCP ligands by NPY [15] could not be replicated in another laboratory [18]. Nevertheless, other studies still suggest interactions between NPY and NMDA [16]. While this debate is not yet settled, it seems clear that any interactions between NPY and NMDA do not occur via a direct modulation of the ion channel or receptor on the pyramidal cells of hippocampus.

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Chapter 3

Neuropeptide Y reduces the frequency of spontaneous but not miniature excitatory postsynaptic currents in CA3 pyramidal cells of rat hippocampus

Introduction

Neuropeptide Y (NPY), originally isolated from porcine brain in 1982 (Tatemoto, Carlquist and Mutt, 1982) is present in high concentrations in many areas of both the central (Hendry, 1993) and peripheral (Sundler, Böller, Ekblad and Hakanson, 1993) nervous systems (CNS and PNS). NPY's function and mechanism of action is better understood in the PNS than in the CNS, particularly its role in the sympathetic control of the cardiovascular system (for review see Wahlestedt and Reis, 1993). In the CNS, NPY has been shown to be involved in behaviours such as feeding (Clark, Kalra, Crowley and Kalra, 1984; Levine and Morley, 1984; Stanley and Leibowitz, 1984), circadian rhythms (Albers and Ferris, 1984) and memory retention (Flood, Hernandez and Morley, 1987) and disorders such as depression and anxiety (Wahlestedt and Reis, 1993) . However, the cellular mechanisms of action of NPY in the CNS are less well understood (for review see Colmers and Bleakman, 1994).

The hippocampal formation of the rat has a relatively high concentration of NPY and its receptors in both the CA subfields and the dentate gyrus (Martel, St-Pierre and Quirion, 1986; Köhler, Schultzberg and Radesater, 1987). Studies have shown that NPY inhibits excitatory synaptic transmission onto pyramidal neurons of both areas CA1 and CA3 without altering the passive membrane properties of the postsynaptic neurons (Colmers, Lukowiak and Pittman, 1987, Haas, Hermann, Greene and Chan-Palay, 1987; McQuiston and Colmers, 1992; Klapstein and Colmers, 1993). Inhibitory synaptic transmission onto the principal neurons of the hippocampal formation is unaffected by NPY (Klapstein and Colmers, 1993). Synaptic transmission onto dentate granule cells is also unaffected by NPY; however, NPY has recently been shown to inhibit N-type Ca^{2+}

channels in dentate granule cells (McQuiston, Petrozzino, Connor and Colmers, 1994). The action of NPY has been the most extensively studied at the Schaffer-collateral-CA1 synapse where it inhibits excitatory synaptic transmission via the activation of a Y_2 receptor (Colmers *et al.* 1987; Colmers, Klapstein, Fournier, St-Pierre and Treherne, 1991).

Previous studies have shown that NPY inhibits commissural, mossy fiber and associational excitatory synaptic transmission onto CA3 pyramidal cells (Klapstein and Colmers, 1993). However, the site and mechanism of NPY's action in CA3 is unknown. We have chosen to investigate the mechanism of NPY inhibition of excitatory synaptic transmission onto CA3 pyramidal neurons. By examining the miniature and spontaneous excitatory postsynaptic currents (Fatt and Katz, 1952; Korn, Burnod and Faber, 1987; Bekkers and Stevens, 1989; Mintz, Gotow, Triller and Korn, 1989; Korn and Faber, 1990; Mintz and Korn, 1991) of CA3 pyramidal cells in acutely-prepared rat hippocampal slices, we can determine the site of NPY inhibition and provide clues to the mechanism by which it acts. Our results suggest that NPY acts presynaptically to inhibit activity-dependent, but not -independent, glutamate release via the activation of a Y_2 receptor.

Methods

Preparation of slices

Slices were prepared from hippocampi of male Sprague-Dawley rats 21 to 35 days old. Rats were anaesthetized with halothane and sacrificed by decapitation. The brain was rapidly removed and placed in ice cold (4°C) artificial cerebral spinal fluid (aCSF) containing (in mM): NaCl 126, KCl 2.5, $MgCl_2$ 2, $CaCl_2$ 2, NaH_2OPO_4 1.25, $NaHCO_3$ 26, glucose 10, saturated with 95% O_2 / 5% CO_2 (carbogen). The brain was either hemisected sagitally and transverse slices (500 μm) of the brain including the hippocampus were cut on a vibratome (TPI, St. Louis, MO) or the hippocampus was dissected free and cut into 450 to 600 μm transverse slices with a tissue chopper. Slices were maintained in carbogenated aCSF at 30°C in a holding chamber for 1 to 2 hours before recording.

Electrophysiological recording

Hippocampal slices were transferred onto a nylon mesh and submerged in a recording chamber continuously perfused with aCSF (35±0.2°C). Spontaneous (sEPSC) and miniature (mEPSC) excitatory postsynaptic currents were recorded from CA3 pyramidal neurons using the "blind" whole cell patch clamping technique (Blanton, Lo Turco, and Kriegstein, 1989; McQuiston and Colmers, 1992). Glass electrodes were pulled on a two-stage puller (PP-83, Narashige) from borosilicate glass (1.5 mm OD, 1.12 mm ID, WPI, Florida) and had initial resistances in the bath of 3 to 6 MΩ when filled with an internal solution of the following composition (in mM): K gluconate 100, KSO_4 15, KCl 15, NaCl 10, EGTA 11, $CaCl_2$ 1, MgATP 2, Na_2GTP 0.3, HEPES 10, pH 7.2 adjusted with KOH, 295 to 305 mOsm. Junction potentials were corrected in the bath before a seal was formed (2 to 10 $G\Omega$). All experiments were performed in the presence of 100 μM picrotoxin (PcTX) to block spontaneous inhibitory postsynaptic currents (sIPSC) caused by the activation of $GABA_A$ currents. mEPSCs were isolated by additionally perfusing the slice with 1 μM tetrodotoxin (TTX) to block impulse-dependent sEPSCs. In some slices, $CdCl_2$ (200 μM) was used instead of or in addition to TTX to examine whether TTX was completely eliminating Ca^{2+}-dependent release. Drugs were applied by bath superfusion, complete exchange taking less than one minute. NPY and [ahx^{5-24}]NPY (Beck-Sickinger, Grouzmann, Hoffmann, Gaida, Van Meir, Waeber, and Jung, 1992) were applied at concentrations of 1 μM and 3 μM respectively. [ahx^{5-24}]NPY, although somewhat less potent than NPY, was used because of its greatly increased diffusion rate in tissue, especially during washout.

sEPSCs and mEPSCs were recorded from CA3 pyramidal neurons voltage clamped at a holding potential of -60 to -90 mV using the continuous voltage clamp mode of an Axoclamp 2A amplifier (Axon Instruments, Burlingame, CA). Currents were filtered at 1 to 3 kHz and digitized at 10 to 25 kHz on a TL-1 interface with a 486-based computer. Parts of the experiment were also recorded on PCM tape (Neurodata, DR 866) at 44 kHz for offline analysis. The access resistance of the cell was frequently monitored by measuring the amplitude of the capacitative current transients from 10 mV hyperpolarizing voltage steps. Changes in access resistance greater than 10 % resulted in artefactual changes in the amplitude and interevent interval distributions of sEPSCs and mEPSCs. Data from cells having such changes were discarded.

Analysis of sEPSCs and mEPSCs

Two different software packages were used to detect sEPSCs and mEPSCs.

SCAN (Strathclyde software, Dagan Corp) is a commercially available program and the other, written by Ankri, Legendre, Faber and Korn (1994) has been described in detail elsewhere. The SCAN package detects events based on amplitudes exceeding a threshold set just above the noise. All detected events were re-examined and rejected or accepted based on subjective visual examination. The program then measured amplitudes and intervals between successive detected events. The second program was used to limit the subjective determination of detected events. The second program detected sEPSCs or mEPSCs by measuring the derivative of the membrane current over a set duration of time. The event could then be accepted or rejected on expected sEPSC or mEPSC shape parameters (ie. peak amplitude, time to peak, and decay time constant). Both programs produced similar results and the analyses using both programs were therefore pooled together. Axum (Trimetrix) was used to further analyze the data and generate figures. Cumulative probability plots were constructed in order to compare the effects of NPY on the distribution of amplitudes and interevent intervals from sEPSCs or mEPSCs. Cumulative probability plots rank the individual amplitudes or intervals in order of increasing magnitude and plot this rank value against the amplitude or interval size. Comparisons between control and drug distributions of sEPSC or mEPSC amplitudes and intervals were made by performing Kolmogorov-Smirnov tests. Distributions were considered different if $P < 0.01$. This analysis is more rigorous than simply examining mean amplitude and intervals because cumulative probability plots and the Kolmogorov-Smirnov test compares the entire distribution of amplitudes or intervals (Van der Kloot, 1991). Mean amplitudes and mean interevent intervals of sEPSCs or mEPSCs under different recording conditions were compared using a Wilcoxon signed-ranks, Kruskal-

Wallis or a Mann-Whitney test; differences of $P < 0.05$ were considered significant. Values given are the mean +/- SEM. Distributions and mean values for the amplitudes and intervals of sEPSCs or mEPSCs were constructed from the analysis of typically 300 to 2000 events detected from approximately 5 minutes of continuous recording.

Solutions and Drugs

Neuropeptide Y (NPY) was obtained from Richelieu Biotechnology Inc., the Y_2-selective agonist [ahx$^{5-24}$]NPY was a kind gift of Dr. A.G. Beck-Sickinger and CNQX was obtained from Tocris (Bristol, UK). All other compounds were obtained from SIGMA (St Louis, MO, USA).

Results

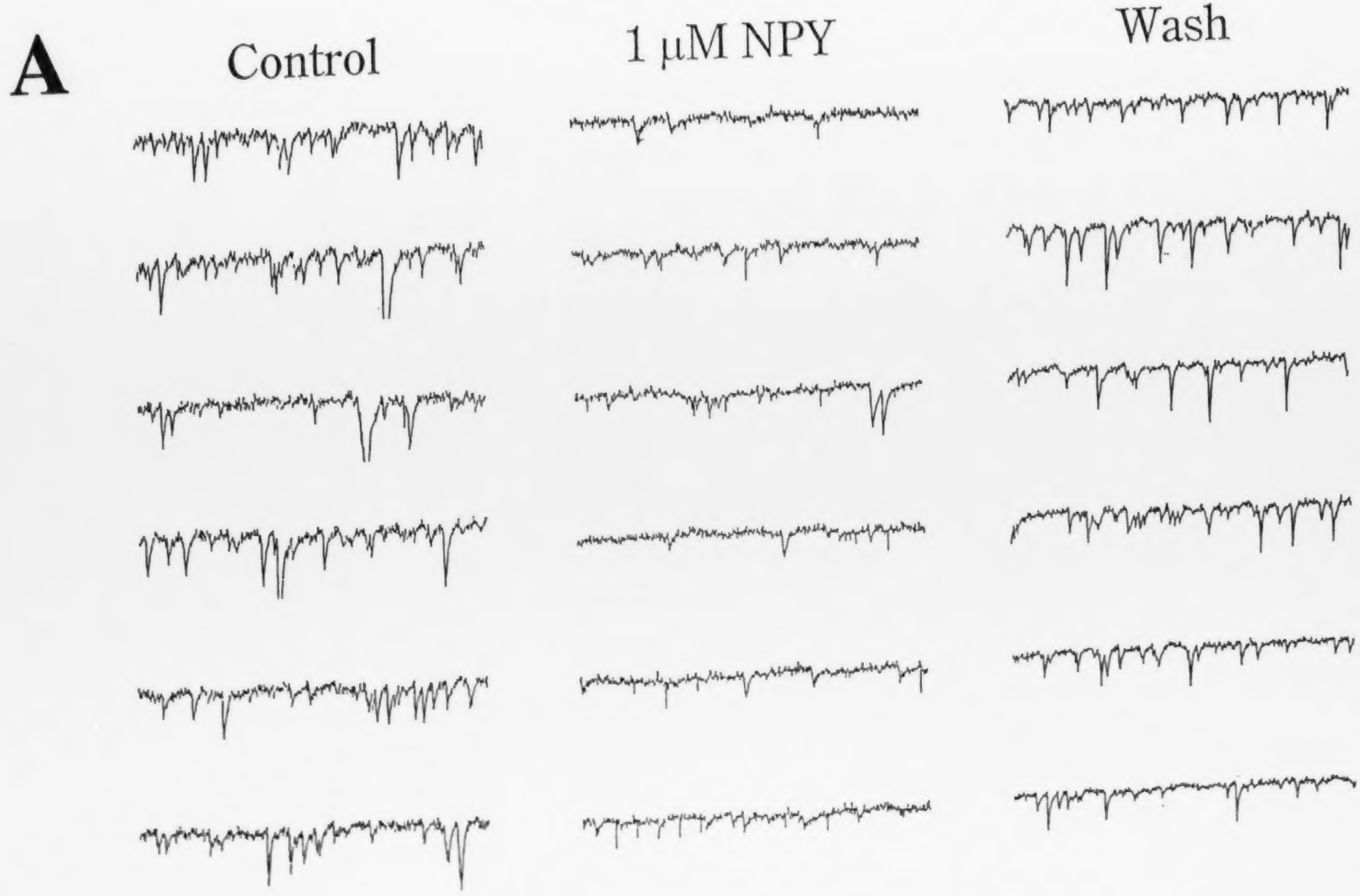
Neuropeptide Y_2 receptor activation inhibits spontaneous excitatory postsynaptic currents

We recorded from 124 CA3 pyramidal cells in 41 hippocampal slices. Only 34 of these cells maintained stable access resistance throughout the experiment and were subsequently analysed. Cells were acquired in current clamp mode and had resting potentials more negative than -60 mV. Spontaneous excitatory and inhibitory postsynaptic potentials were immediately obvious before voltage clamping the cell and beginning the experiment. Spontaneous excitatory postsynaptic currents (sEPSCs) in CA3 pyramidal neurons of rat hippocampal slices were isolated from spontaneous inhibitory postsynaptic currents (sIPSCs) by the inclusion of the 100 μM PcTX in the aCSF perfusing the slice. These sEPSCs were shown to be the result of the activation of glutamate receptors, as application of the ionotropic glutamate receptor antagonists CNQX (10 μM) and APV (50 μM) eliminated all such currents.

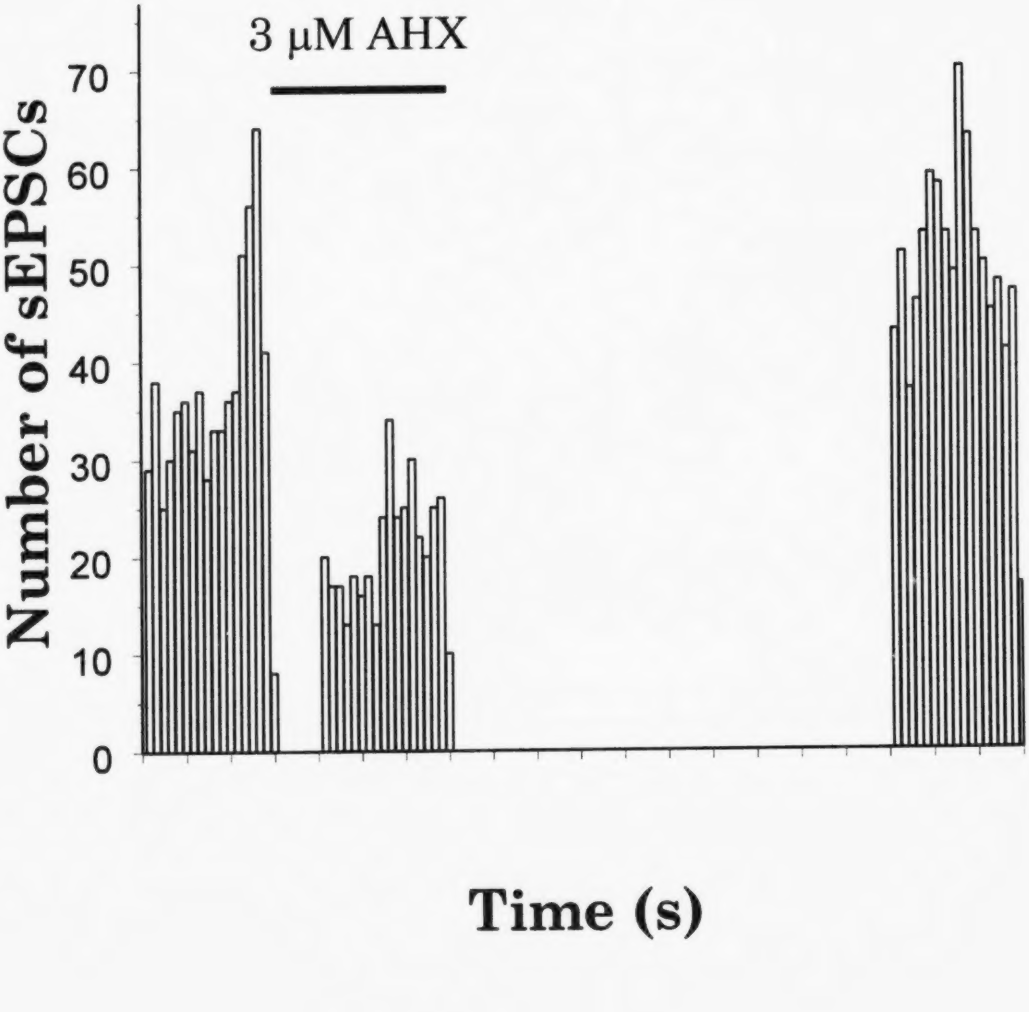
A typical whole cell patch clamp recording of these sEPSCs is illustrated in Figure 3-1A. From these raw data traces it can be seen that NPY (1 μM) dramatically reduces the frequency of sEPSCs. Figure 3-1B shows data from another cell where the sEPSC frequency is plotted against time. The Y_2-selective agonist [ahx$^{5-24}$]NPY (3 μM) reversibly decreased the frequency of sEPSCs detected. The mean sEPSC amplitude of the cell in Figure 3-1B was also reversibly reduced by [ahx$^{5-24}$]NPY from 46.9 ± 0.2 pA under control to 41.4 ± 0.5 pA (Fig. 3-1C, $P < 0.0001$, Kruskal-Wallis test). Because NPY and [ahx$^{5-24}$]NPY produced similar results at the concentrations used, data from experiments using both agonists were analyzed together. It should also be noted that the effect of [ahx$^{5-24}$]NPY developed more rapidly (typically 1 to 2 min) and reversed much

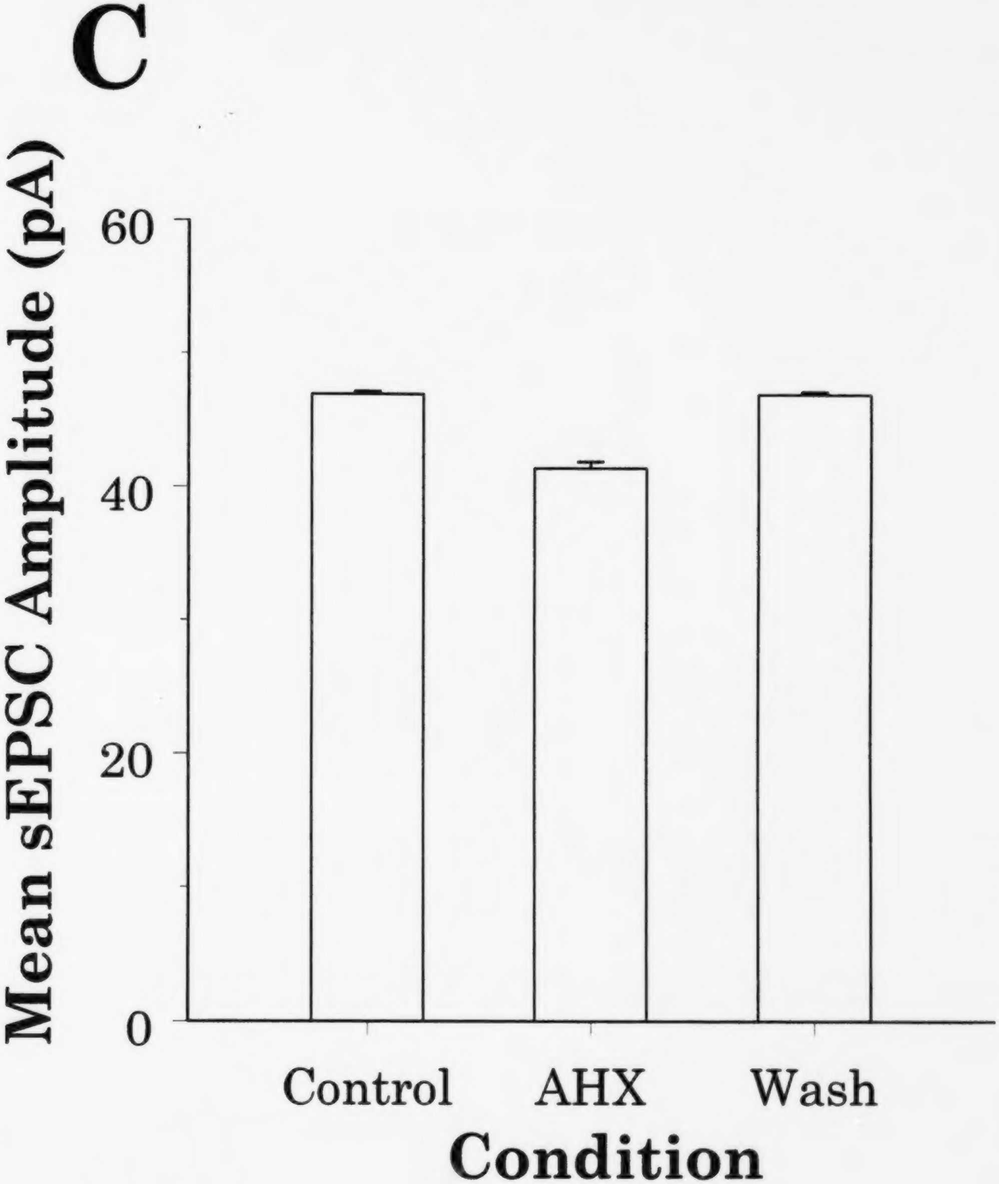
Figure 3-1. NPY and the Y$_2$ agonist, [ahx$^{5-24}$]NPY, decrease the frequency and amplitude of sEPSCs in CA3 pyramidal neurons

A. Consecutive continuous traces of sEPSCs from a CA3 pyramidal neuron under control conditions (left), treated with 1 μM NPY (middle), and following recovery (right). Scale bar 500 ms horizontal, 50 pA vertical. **B**. Data from a different CA3 cell. The number of sEPSCs detected in 10 s bins throughout the experiment is displayed. Gaps in the histogram represent periods during which data was not recorded. The Y$_2$ agonist, [ahx$^{5-24}$]NPY (3 μM) was applied during the time indicated by the overlying bar. A horizontal division is equal to 60 s. [ahx$^{5-24}$]NPY decreases the number of sEPSCs detected during the 10 s bins. **C**. A bar graph of the mean amplitude of the sEPSCs from the same CA3 pyramidal cell in B. The mean amplitude of the sEPSCs when treated with [ahx$^{5-24}$]NPY (41.4 ± 0.5 pA) was significantly smaller than the mean amplitudes of the sEPSCs under control (46.9 ± 0.2 pA) and wash (46.9 P 0.2 pA) ($P < 0.0001$, Kruskal-Wallis test).


A
Control
1 μM NPY
Wash

B




C
Mean sEPSC Amplitude (pA)
60
40
20
0
Control
AHX
Wash
Condition

more rapidly upon washout (10 to 20 min) than NPY itself, which typically takes 5 to 10 min to take effect and up to 60 min to completely recover following wash (Colmers, Lukowiak and Pittman, 1985, 1987, 1988).

We next examined the effect of NPY and the selective Y_2 agonist on sEPSC amplitude and sEPSC interval distributions, by constructing cumulative probability plots. Comparisons between distributions in control, NPY and wash were made by a two-sample Komolgorov-Smirnov test (K-S test) (Van der Kloot, 1991). A typical example of such an analysis is shown in Figure 3-2. In this cell, NPY (1 μM) shifts the sEPSCs to larger interval ranges (Fig. 3-2B, $P < 0.0001$ K-S test) and to smaller amplitude ranges (Fig. 3-2A, $P < 0.0001$ K-S test). In 10 other cells, similar changes in the sEPSC interval and amplitude distributions were observed. However, in 5 cells a shift in the sEPSC interval distribution to longer intervals was observed without a significant change in the sEPSC amplitude distribution.

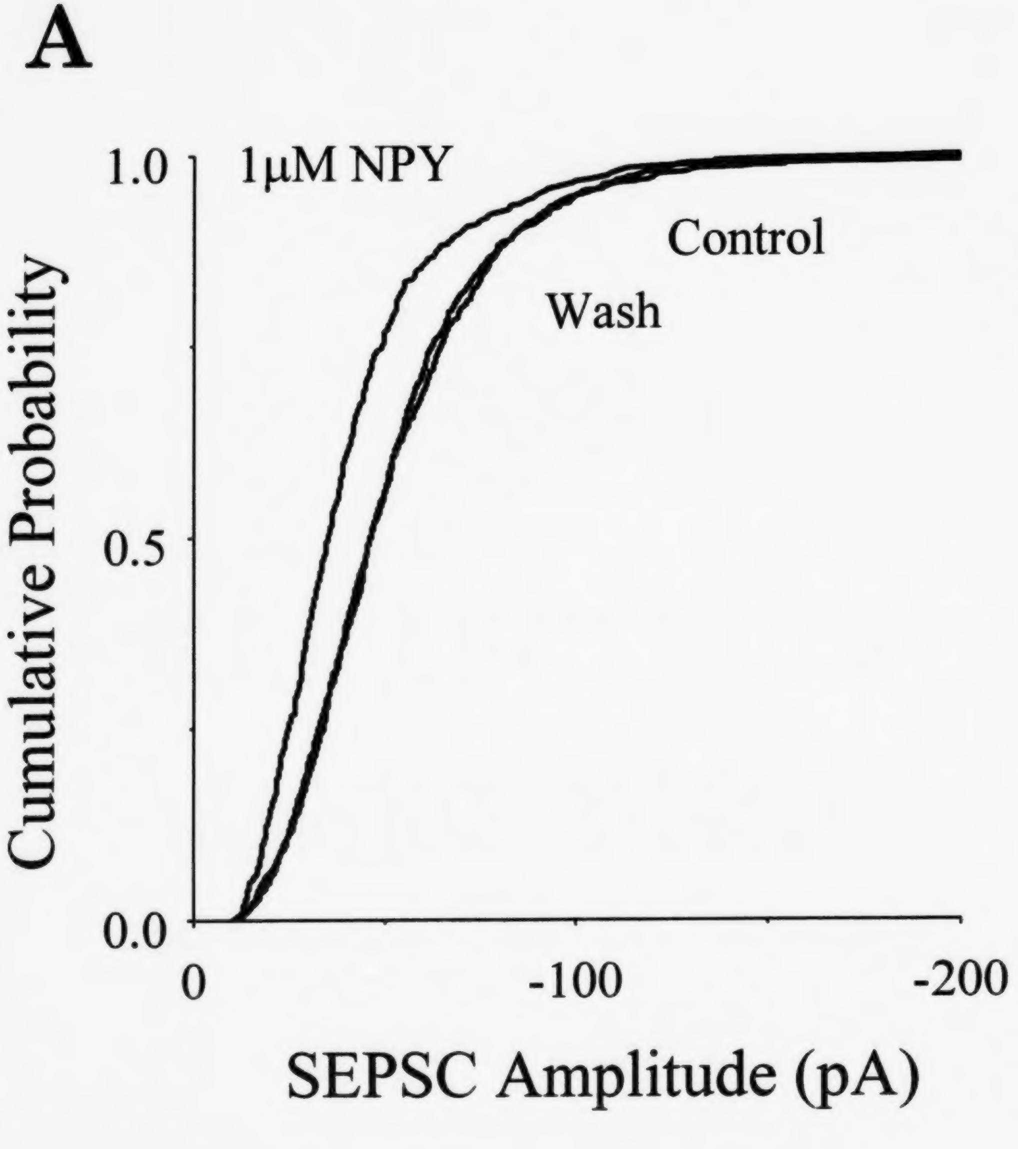
NPY inhibits only activity-dependent excitatory synaptic transmission

The effect of NPY and [ahx$^{5-24}$]NPY on mEPSCs was examined in order to determine whether NPY also acts postsynaptically to inhibit excitatory synaptic transmission.

TTX (1 μM) was added in the presence of PcTX to the perfusate to eliminate EPSCs arising from presynaptic impulses. mEPSCs are thought to result from the spontaneous release of transmitter quanta in a manner independent of presynaptic Ca^{2+} influx (Fatt and Katz, 1952). Figure 3-3A is an example of mEPSCs recorded under these conditions. These raw data traces show that NPY (1 μM; left) appears to have no effect on the frequency or amplitude of mEPSCs compared to control (right) (Fig. 3-3A).

Figure 3-2. NPY modulates sEPSC amplitude and interevent interval distributions from a CA3 pyramidal neuron

A. Cumulative probability plot of the distribution of sEPSC amplitudes. NPY (1 μM) shifts the sEPSCs to smaller amplitudes compared to control and wash ($P < 0.0001$, K-S test). **B**. Cumulative probability plot of the distribution of sEPSC intervals. NPY (1 μM) causes a shift to longer intervals compared to control and wash ($P < 0.0001$, K-S test).


A
1.0
0.5
0.0
1μM NPY
Control
Wash
Cumulative Probability
0
-100
-200
SEPSC Amplitude (pA)

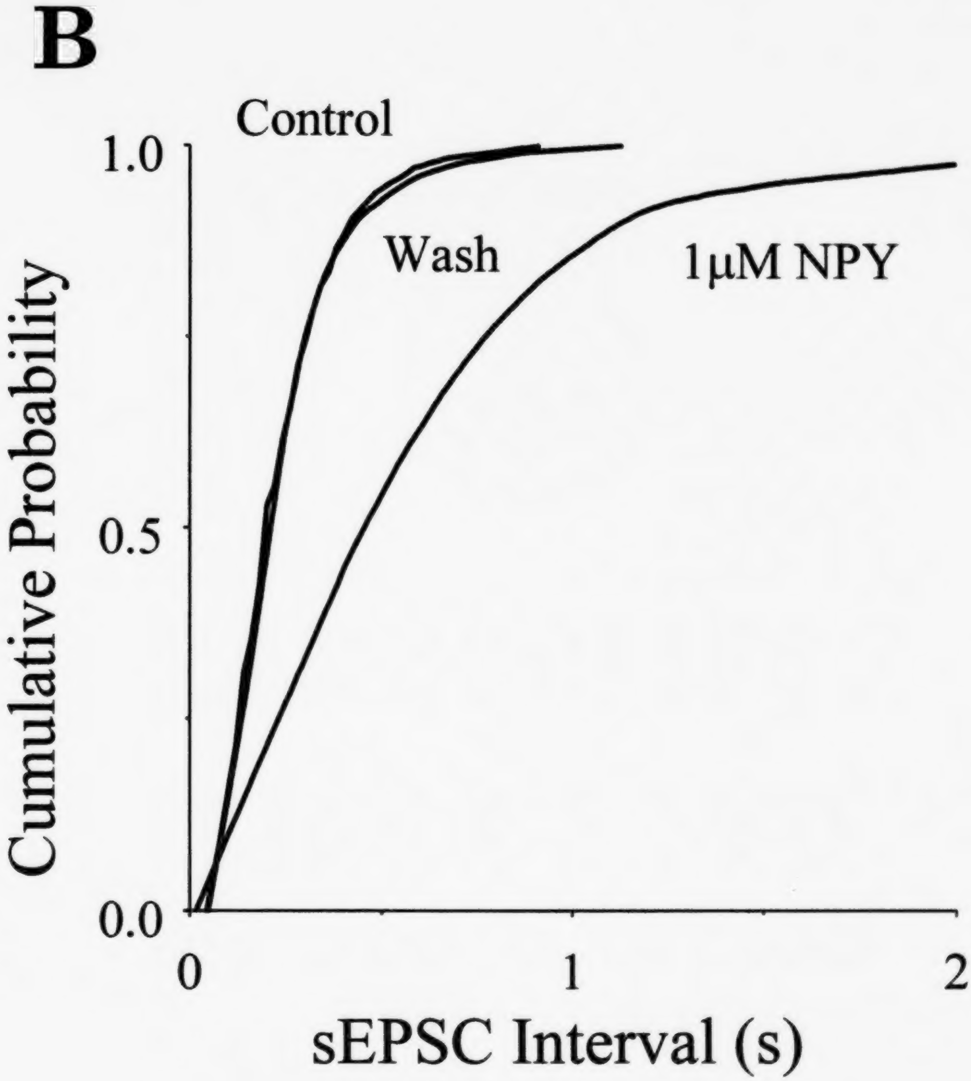
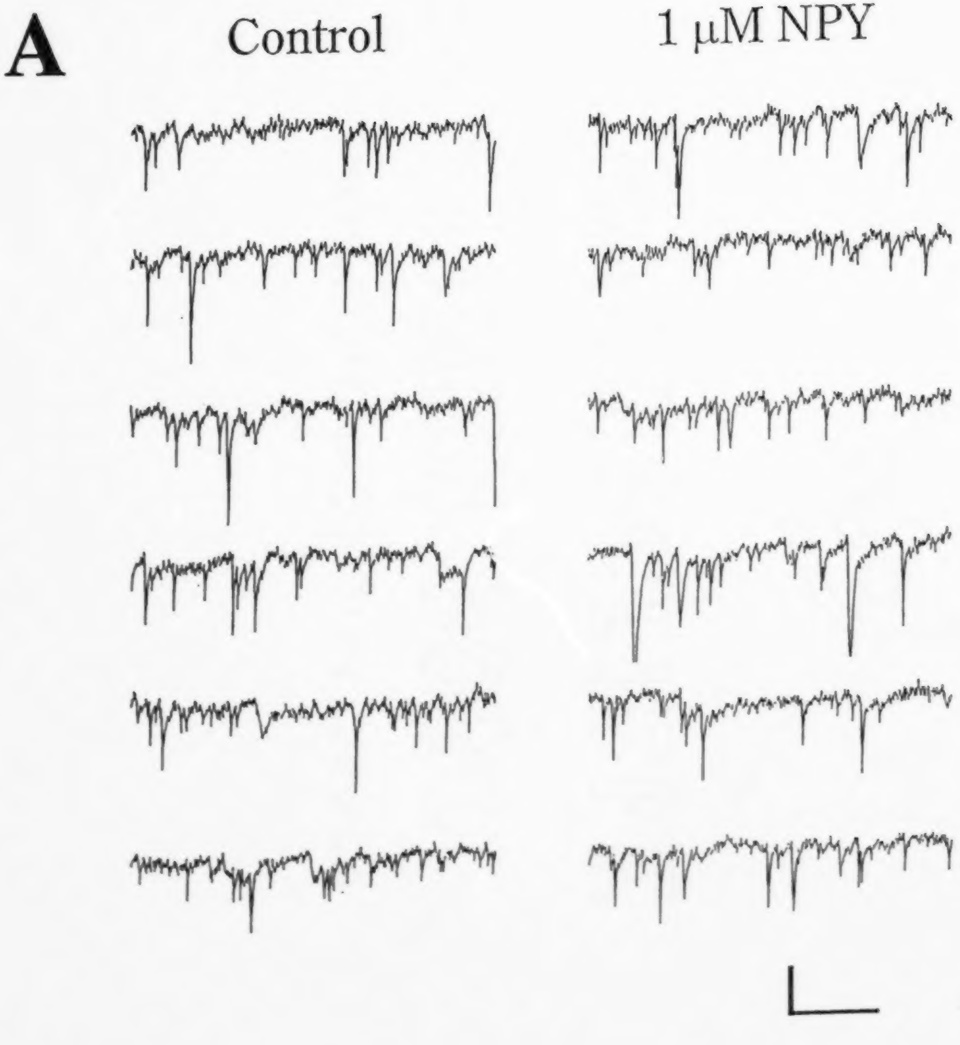
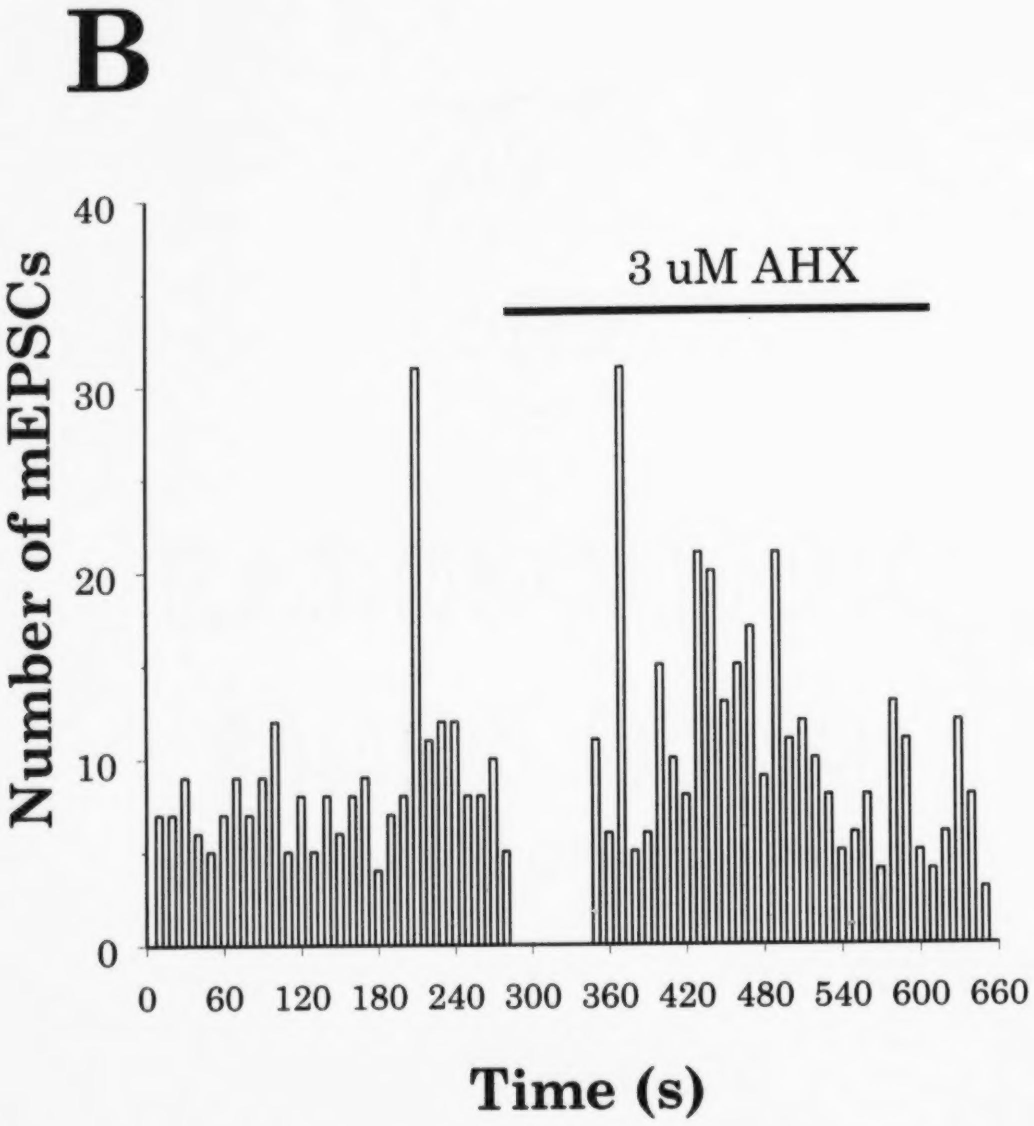
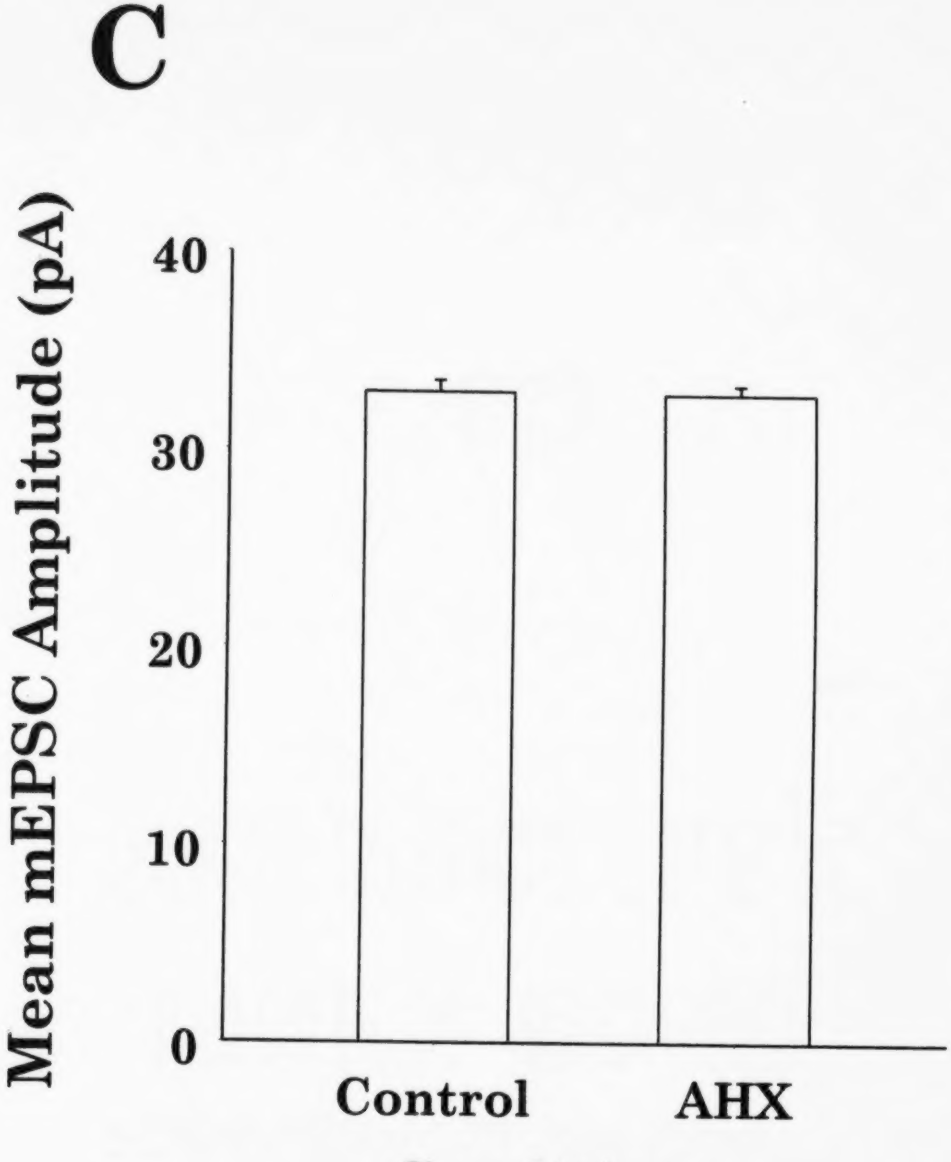

B
Control
Wash
1μM NPY
Cumulative Probability
1.0
0.5
0.0
0
1
2
sEPSC Interval (s)

Figure 3-3. NPY and the Y_2 agonist $[ahx^{5-24}]$NPY do not affect the frequency or amplitude of mEPSCs recorded from a CA3 pyramidal neuron

A. Consecutive continuous traces of mEPSCs recorded in 1 μM TTX from a CA3 pyramidal neuron under control conditions (left), and in the presence of 1 μM NPY (right). Scale bar 500 ms horizontal, 50 pA vertical. **B**. Data from a different cell. A histogram of the number of mEPSCs detected in 10 s bins throughout the experiment is shown. A horizontal division is equal to 60 s. $[ahx^{5-24}]$NPY (3 μM) was applied during the time indicated by the overlying bar. $[ahx^{5-24}]$NPY did not appear to reduce the number of mEPSCs detected during the 10 s bins. **C**. A bar graph of the mean mEPSC amplitude from the same CA3 pyramidal cell in B. The mean amplitude of the mEPSCs was not significantly different between $[ahx^{5-24}]$NPY (32.8 ± 0.6 pA) and control (32.9 ± 0.5 pA, $P > 0.39$, Mann-Whitney test).


A
Control
1 μM NPY


B
40
30
20
10
0
Number of mEPSCs
3 uM AHX
0
60
120
180
240
300
360
420
480
540
600
660
Time (s)


C
Mean mEPSC Amplitude (pA)
40
30
20
10
0
Control
AHX
Condition

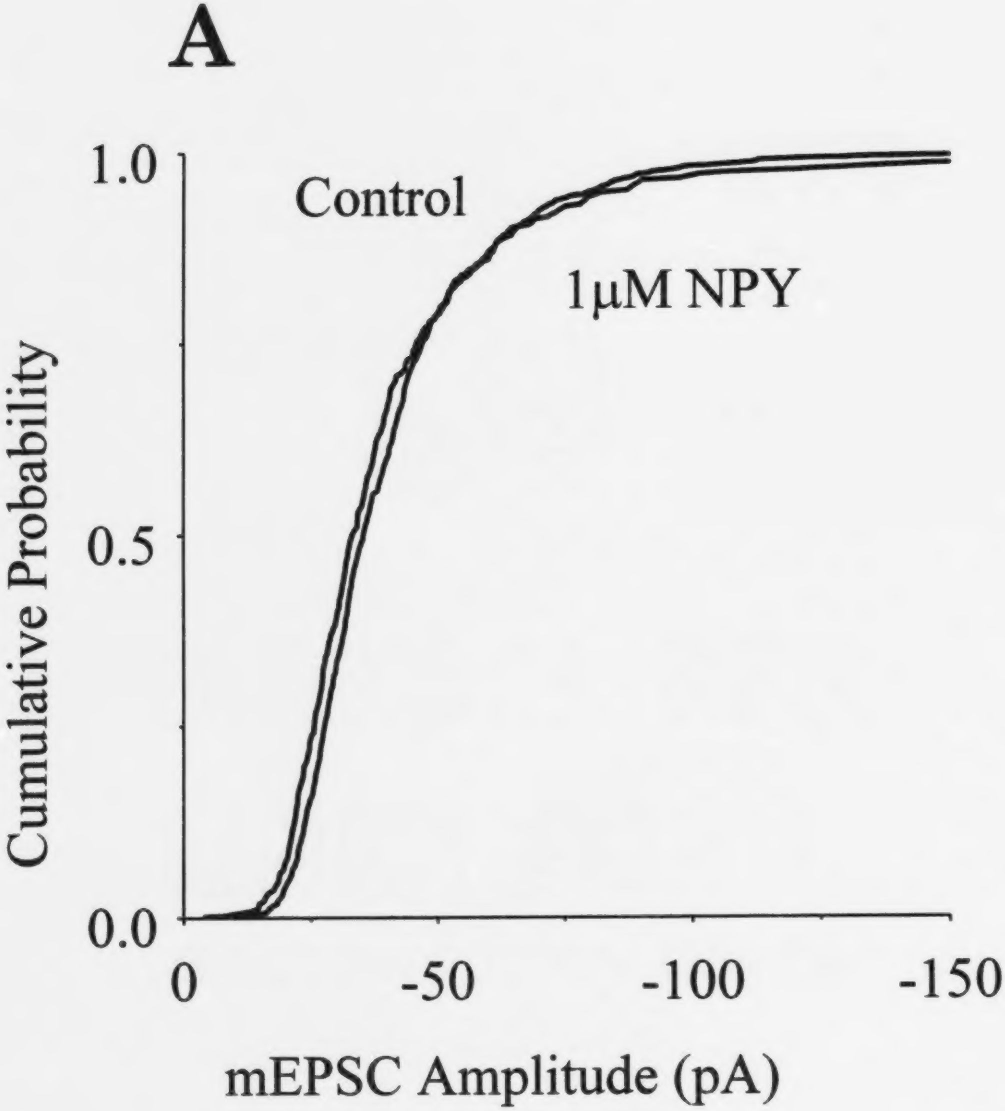
The rapid rate of washout of [ahx$^{5-24}$]NPY permitted us to examine the effect of Y_2 receptor activation on both sEPSCs and mEPSCs in the same cell ($N = 7$). In such an experiment, [ahx$^{5-24}$]NPY (3 μM) did not decrease the frequency of mEPSCs (Fig. 3-3B), in contrast to the decrease in the sEPSC frequency observed in the same cell with [ahx$^{5-24}$]NPY (Fig. 3-1B). Furthermore, [ahx$^{5-24}$]NPY did not affect the mean amplitude of mEPSCs (Fig. 3-3C: control 32.9 ± 0.6 pA: [ahx$^{5-24}$]NPY 32.8 ± 0.5 pA: $P > 0.39$ Mann-Whitney test) although it produced a decrease in the mean sEPSC amplitude of the same cell (Fig. 3-1C).

Cumulative amplitude and interval distributions of the mEPSC data in control and in NPY were compared using the Kolmogorov-Smirnov test (K-S test). The mEPSC interval distribution was unchanged by NPY (Fig. 3-4B, $P > 0.99$), nor did it affect the amplitude distribution (Fig. 3-4A, $P > 0.06$). Neither NPY nor [ahx$^{5-24}$]NPY affected the interval and amplitude distributions of mEPSCs in 14 further neurons.

In 7 neurons, the effect of [ahx$^{5-24}$]NPY was studied on both sEPSCs and mEPSCs. in 6 of these cells, [ahx$^{5-24}$]NPY caused a shift to smaller events in the sEPSC amplitude distribution. In 5 such cells, application of TTX was also sufficient to cause a significant shift to smaller sEPSC amplitude distribution (Fig. 3-5A, B). The cell of Fig. 3-5 shows that application of TTX significantly shifts the distribution of sEPSC amplitudes to smaller ranges compared to either in control or in [ahx$^{5-24}$]NPY (K-S test, $P < 0.001$). Similar results were found in the 4 other cells; the mean quantal EPSC amplitudes of the 5 cells are plotted in Fig. 3-5B. This bar graph shows that both [ahx$^{5-24}$]NPY and TTX decrease the mean sEPSC amplitude ($P < 0.05$, Kruskal-Wallis test). The mean sEPSC amplitude in TTX was significantly smaller than in the presence

Figure 3-4. NPY does not affect mEPSC amplitude or interevent interval distributions in a CA3 pyramidal neuron

A. Cumulative probability plot of the distribution of mEPSC amplitudes. NPY (1 μM) does not alter the mEPSC amplitude distribution ($P > 0.06$, K-S test). **B**. Cumulative probability plot of the distribution of mEPSC intervals. NPY (1 μM) does not alter the distribution of mEPSC intervals ($P > 0.99$, K-S test).


A
1.0
0.5
0.0
Cumulative Probability
Control
1μM NPY
0
-50
-100
-150
mEPSC Amplitude (pA)

B

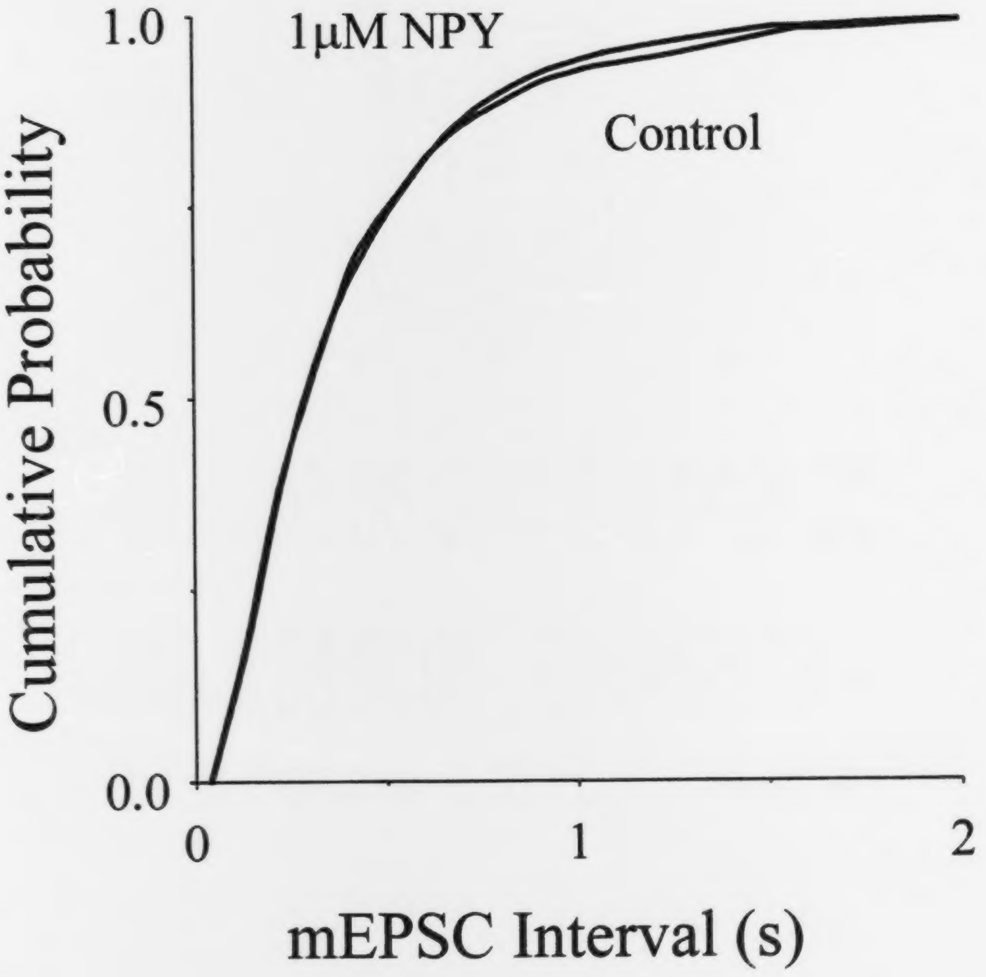
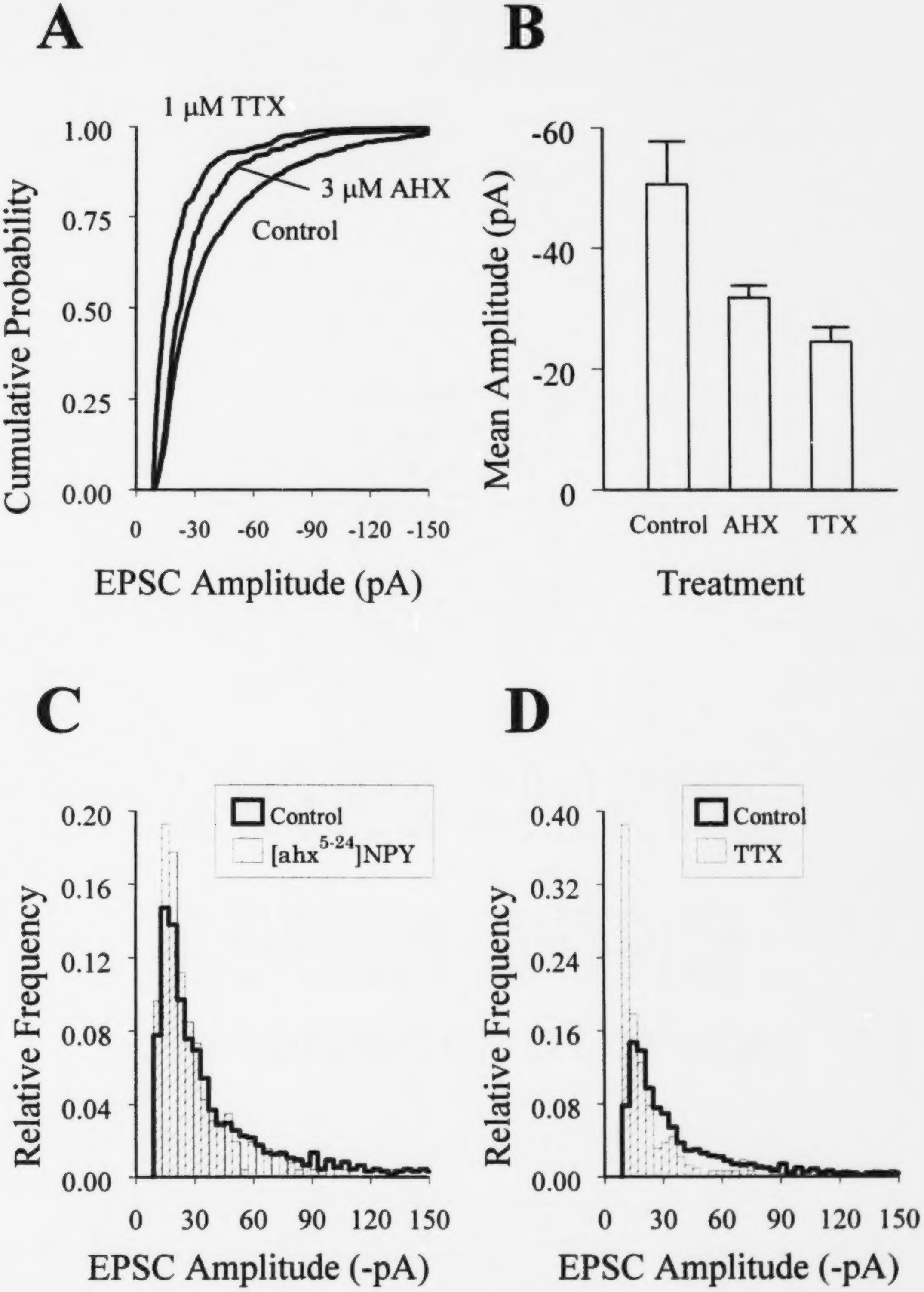

1.0
0.5
0.0
0
1
2
Cumulative Probability
1μM NPY
Control
mEPSC Interval (s)

Figure 3-5. Tetrodotoxin modulates the amplitude distribution of sEPSCs in a CA3 pyramidal neuron

A. [ahx$^{5-24}$]NPY (3 μM) shifted the amplitude distribution of sEPSCs in this neuron to smaller values ($P < 0.001$, K-S test). Following washout of [ahx$^{5-24}$]NPY, TTX (1 μM) also shifted the sEPSC distribution to smaller amplitudes ($P < 0.001$, K-S test). **B**. The effect of [ahx$^{5-24}$]NPY and TTX on the mean sEPSC amplitudes of cells treated with both. Mean sEPSC amplitudes were decreased after the application of [ahx$^{5-24}$]NPY (3 μM) (-31.8 ± 2.1) and TTX (1 μM) (-24.6 ± 2.4) compared to controls (-50.5 ± 7.2, $P < 0.01$, Kruskal-Wallis test, $N = 5$). **C**. A histogram of sEPSC amplitudes (bin width 4 pA) from the data in A. [ahx$^{5-24}$]NPY (3 μM) (hatched bars) reduces the relative frequency of large sEPSCs amplitudes when compared to the control sEPSC distribution (open bars). **D**. A histogram of control sEPSC (open bars) and mEPSC amplitudes (hatched bars) show that TTX (1 μM) reduces the relative frequency of large mEPSC amplitudes compared to control.


A
1 µM TTX
3 µM AHX
Control
Cumulative Probability
1.00
0.75
0.50
0.25
0.00
0
-30
-60
-90
-120
-150
EPSC Amplitude (pA)
B
Mean Amplitude (pA)
-60
-40
-20
0
Control
AHX
TTX
Treatment
C
Control
[ahx^5-24]NPY
Relative Frequency
0.20
0.16
0.12
0.08
0.04
0.00
0
30
60
90
120
150
EPSC Amplitude (-pA)
D
Control
TTX
Relative Frequency
0.40
0.32
0.24
0.16
0.08
0.00
0
30
60
90
120
150
EPSC Amplitude (-pA)

of 3μM $[\mathrm{ahx}^{5-24}]$NPY (Kruskal-Wallis test, $P < 0.05$) (Fig. 3-5B). Amplitude histograms from the data in Figure 3-5A show that 3 μM $[\mathrm{ahx}^{5-24}]$NPY causes a reduction in the relative frequency of large sEPSC amplitudes (Fig 3-5C). Furthermore, 1 μM TTX also reduces the relative frequency of large sEPSC amplitudes when compared to control (Fig. 3-5D).

The effects of NPY and $[\mathrm{ahx}^{5-24}]$NPY on both sEPSCs and mEPSCs are summarized in Figure 3-6. In order to illustrate the effect of Y_2 receptor agonists on all the cells examined, the sEPSC or mEPSC mean amplitudes and intervals were averaged. NPY agonists significantly increased the average mean sEPSC interval from 458 ± 28 ms to 1542 ± 196 ms ($P < 0.001$, Wilcoxon signed-ranks test $N = 16$). However, NPY agonists did not affect the average mean mEPSC interval (control 1196 ± 98 ms, NPY agonist 1255 ± 116 ms, $P > 0.19$ Wilcoxon signed-ranks test, $N = 15$; Fig. 3-6B). Furthermore, NPY agonists decreased the average mean sEPSC amplitude from 64.2 ± 2.0 pA to 44.3 ± 1.9 pA ($P < 0.02$, Wilcoxon signed-ranks test, $N = 16$) whereas NPY agonist had no affect on the average mean mEPSC amplitude (control 41.8 ± 2.4, NPY agonist 41.7 ± 2.3, $P > 0.99$, Wilcoxon signed-ranks test, $N = 15$) (Fig. 3-6A).

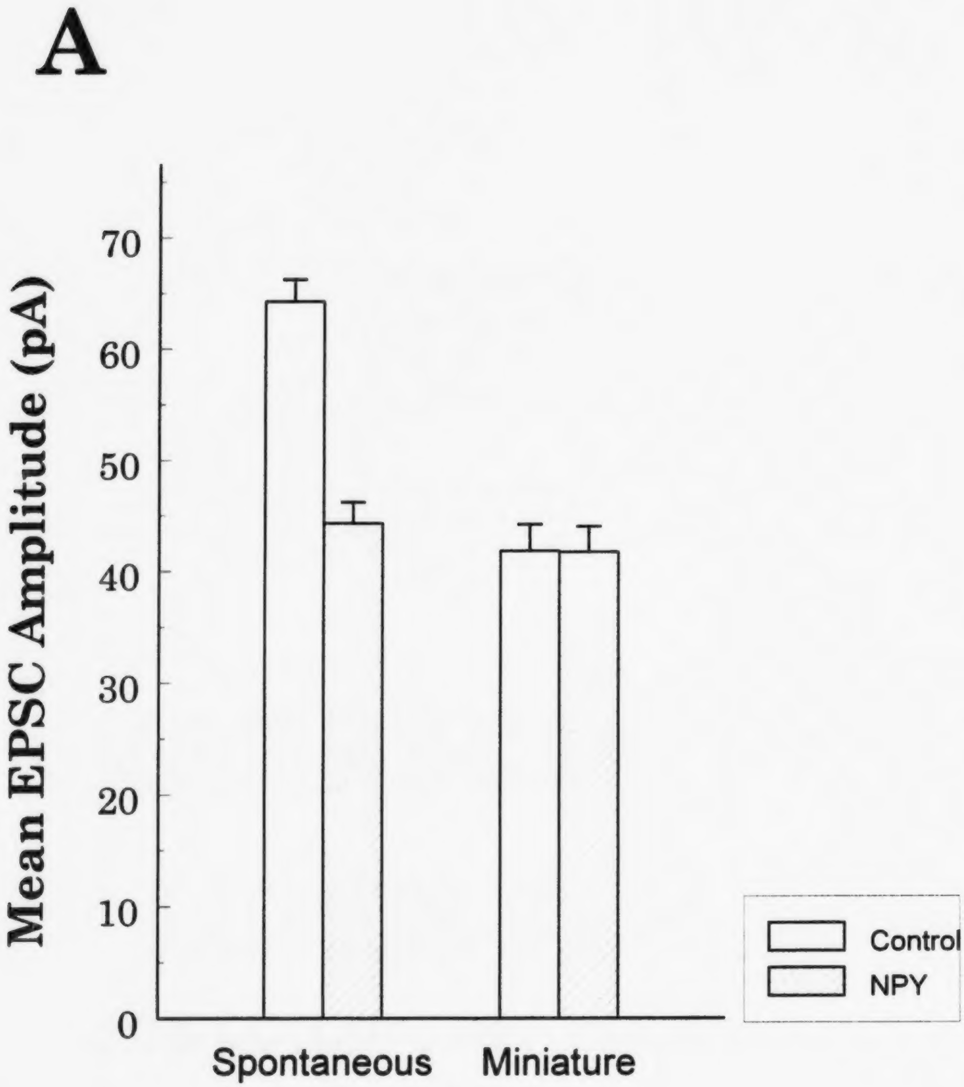
Tetrodotoxin eliminates Ca^{2+}-dependent transmitter release

To determine whether the mEPSCs in TTX are entirely independent of Ca^{2+} influx (Korn, Bausela, Charpier and Faber, 1993), we determined: 1) if the Ca^{2+} channel blocker, Cd^{2+}, could further inhibit mEPSC amplitude or interval distributions in the presence of TTX; and 2) whether NPY had the same effect on the mEPSCs observed when the slice was perfused with Cd^{2+} instead of TTX.

Figure 3-7 shows the effect of 200 μM Cd^{2+} on amplitude and interval

Figure 3-6. Summary of the effect of NPY receptor activation on sEPSCs and mEPSCs of CA3 pyramidal neurons

A. The effect of NPY or [ahx$^{5-24}$]NPY (pooled data) on the mean sEPSC amplitudes (left) and mean mEPSC amplitudes (right). The mean sEPSC amplitudes were significantly reduced by NPY agonist (44.3 ± 1.9 pA) compared to controls (64.2 ± 2.0 pA, $P < 0.05$ Wilcoxon signed ranks test, N = 16). The mean mEPSC amplitudes were unaffected by NPY agonists (41.7 ± 2.3 pA) compared to controls (41.8 ± 2.4 pA, $P > 0.99$ Wilcoxon signed-ranks test, N = 15). **B**. The effect of NPY or [ahx$^{5-24}$]NPY (pooled data) on the mean sEPSC intervals (left) and mean mEPSC intervals (right). The mean sEPSC intervals were significantly increased by NPY agonists (1542 ± 196 ms) compared to controls (457 ± 28 ms, $P < 0.001$, N = 16). The mean mEPSC intervals were not significantly affected by NPY agonists (1196 ±98 ms) compared to controls (1255 ± 116 ms, $P > 0.19$ Wilcoxon signed-ranks test, N = 15).


A
Mean EPSC Amplitude (pA)
0
10
20
30
40
50
60
70
Spontaneous
Miniature
Condition
Control
NPY

B

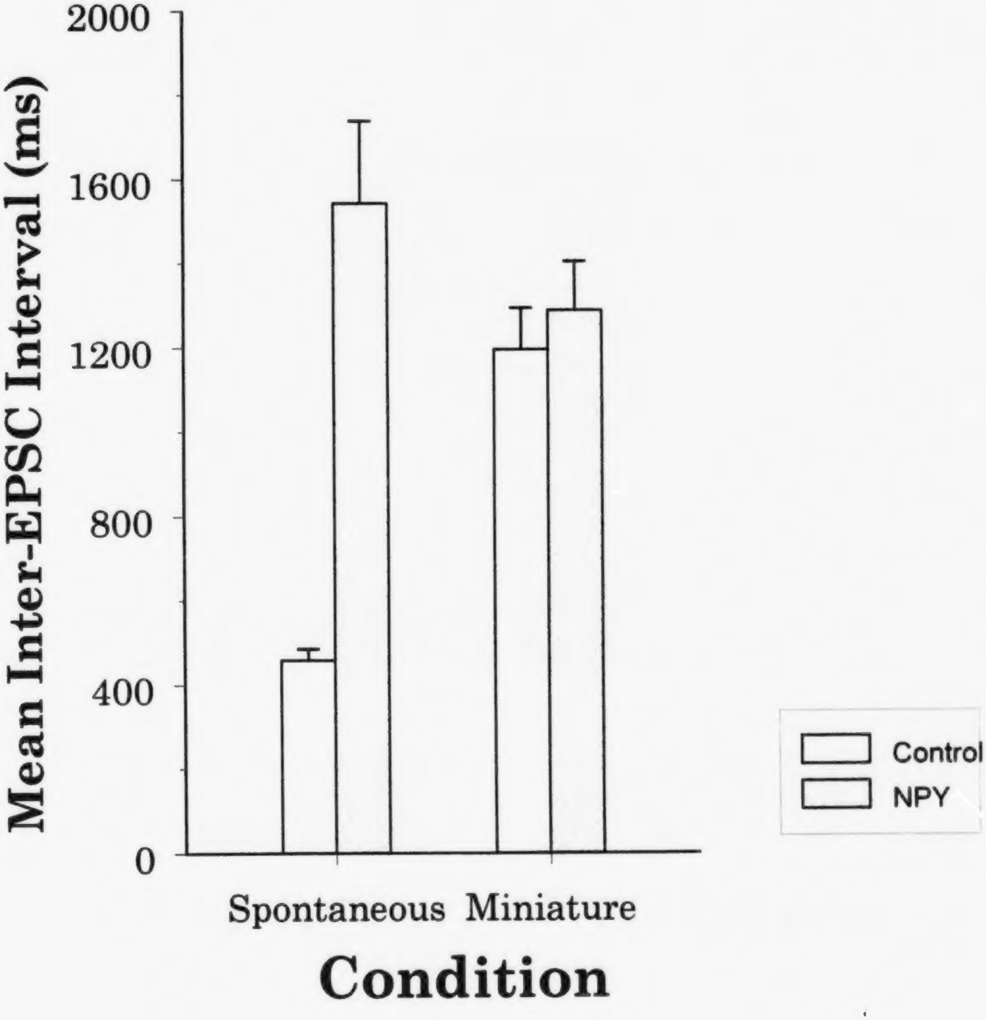
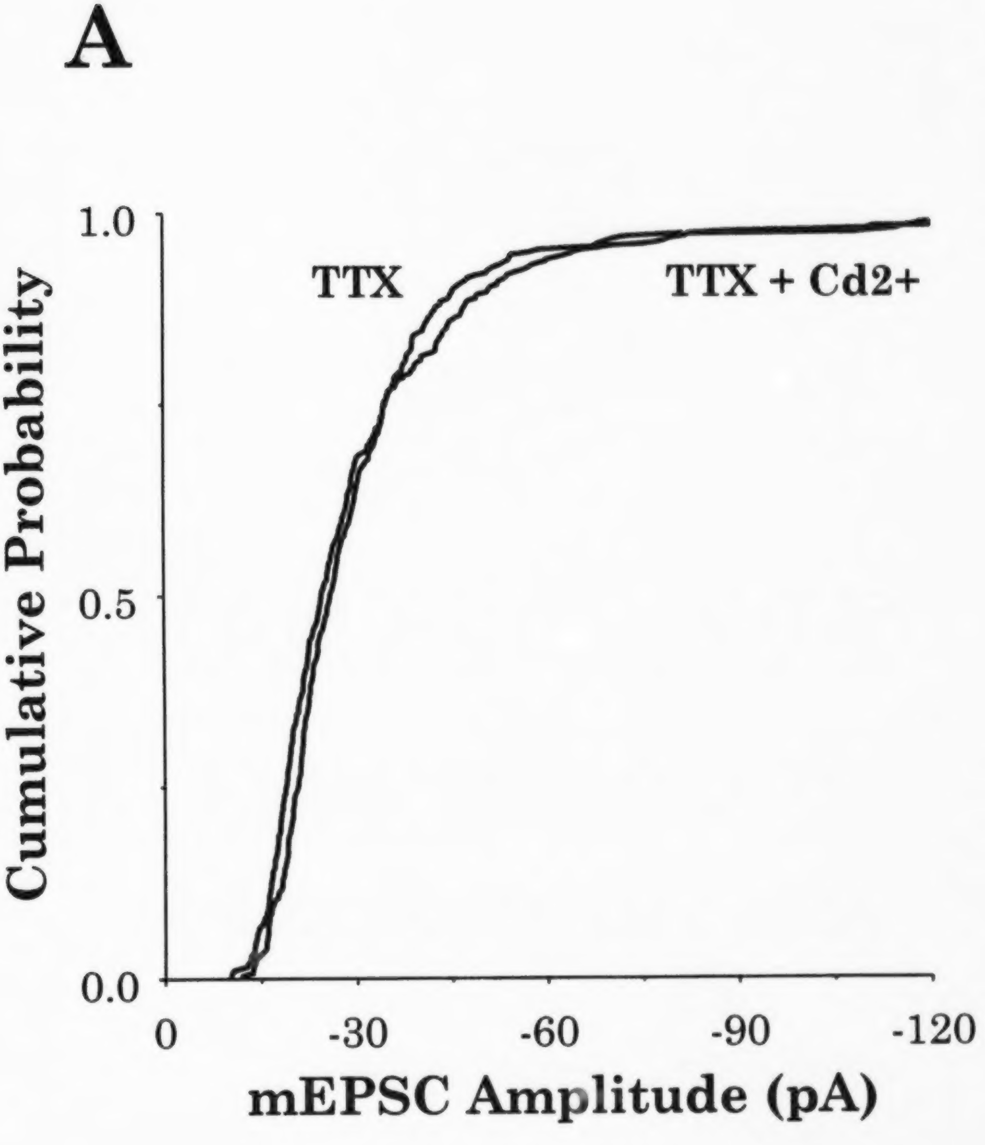
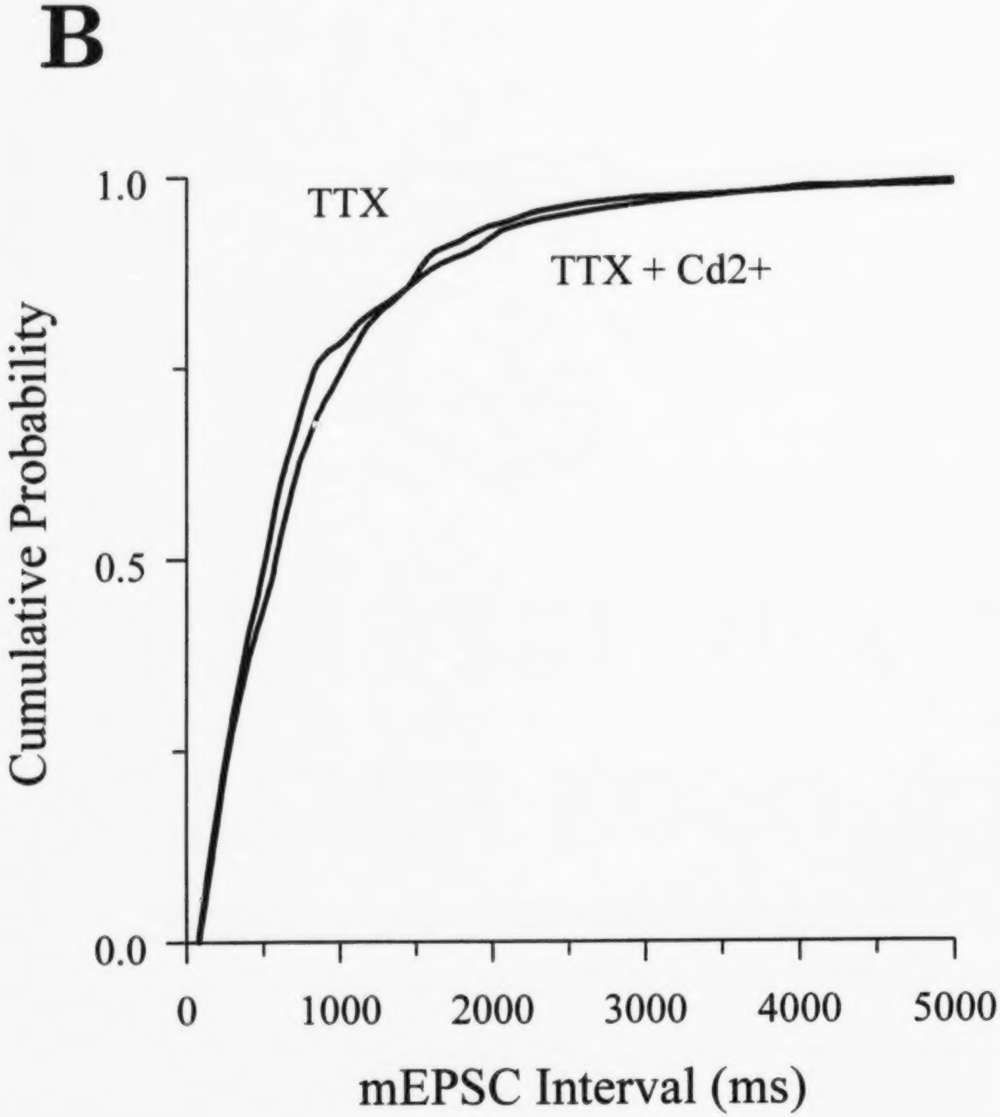

Mean Inter-EPSC Interval (ms)
2000
1600
1200
800
400
0
Spontaneous
Miniature
Condition
Control
NPY

Figure 3-7. Cadmium does not affect mEPSC amplitude or interevent interval distributions from a CA3 pyramidal neuron

A. Cumulative probability plot of the distribution of mEPSC amplitudes. Cadmium (Cd^{2+}) (200 μM) does not alter the mEPSC amplitude distribution ($P > 0.05$, K-S test). **B**. Cumulative probability plot of the mEPSC interval distribution. Cd^{2+} (200 μM) does not alter the mEPSC interval distribution ($P > 0.22$).


A
Cumulative Probability
1.0
0.5
0.0
TTX
TTX + Cd2+
0
-30
-60
-90
-120
mEPSC Amplitude (pA)


B
1.0
0.5
0.0
Cumulative Probability
TTX
TTX + Cd2+
0
1000
2000
3000
4000
5000
mEPSC Interval (ms)

distributions of mEPSCs in 1 μM TTX. In this cell, Cd^{2+} affected neither the amplitude (Fig. 3-7A, $P > 0.05$, K-S test) nor the interval distribution (Fig. 3-7B, $P > 0.22$, K-S test) ($n = 2$). In another cell, mEPSCs were isolated by perfusing the slice with Cd^{2+} (200 μM) rather than TTX. NPY (1 μM) did not affect the distribution of mEPSC amplitudes (Fig. 3-8A, $P > 0.05$, K-S test) or intervals (Fig. 3-8B, $P > 0.61$, K-S test) ($n = 3$).

Detection and analysis can discern selective changes in amplitude and interval distributions

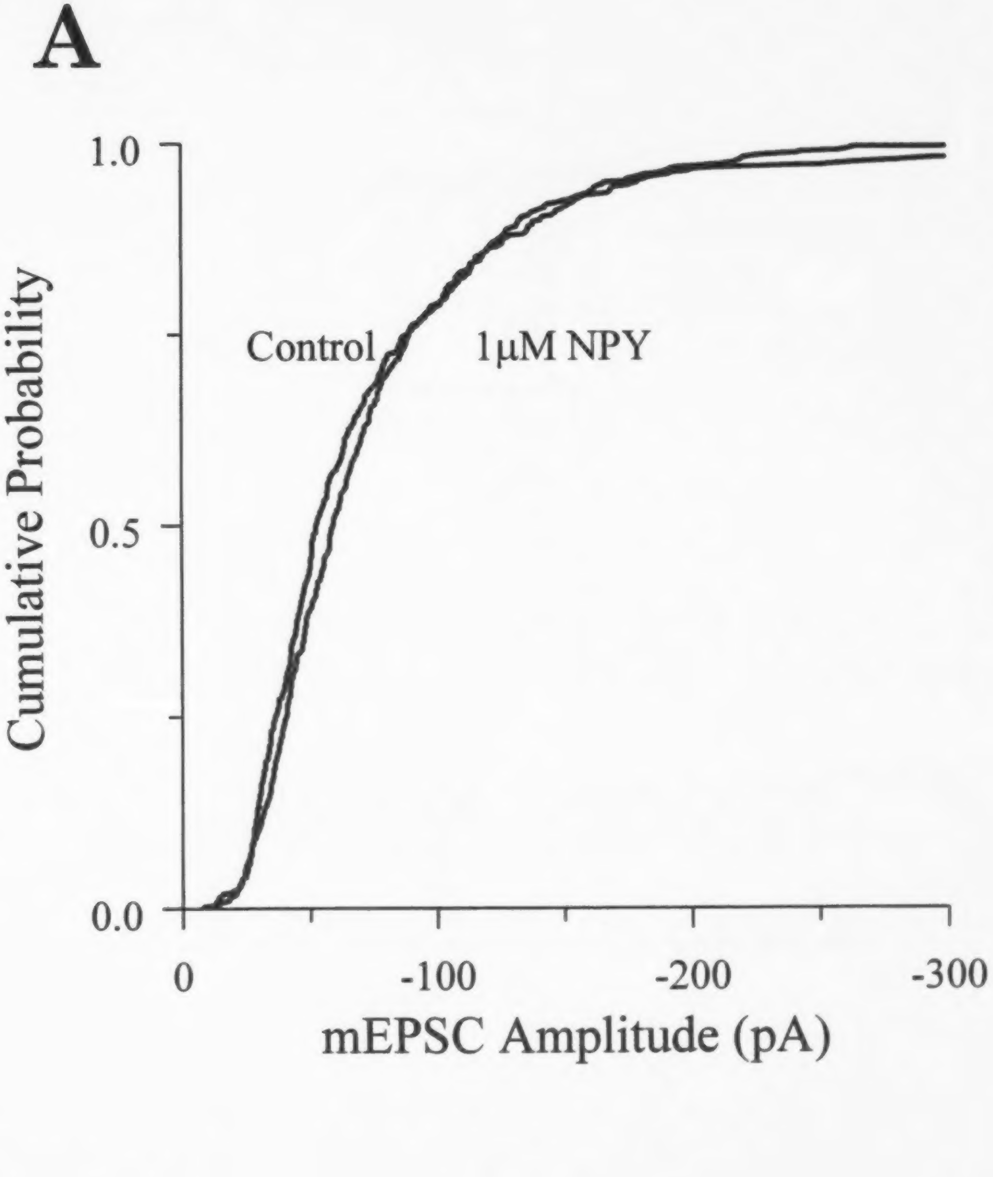
In order to determine whether we can accurately detect a change in amplitude or interval distributions of quantal synaptic events, we selectively altered the amplitude distribution by changing the membrane potential of the cell, and selectively changed the interval distribution by adding sucrose (50 μM) to the perfusate.

When we held the membrane potential at two different voltages, the sEPSC amplitude distribution was shifted to larger amplitudes at more hyperpolarized potentials, as would be expected from the increased driving force (Fig. 3-9A, $P < 0.01$, K-S test). The interval distribution of sEPSCs was not affected by the hyperpolarization (Fig. 3-9B, $P > 0.61$, K-S test). Similar results were obtained for 2 other cells.

Addition of sucrose to increase the osmolarity of the extracellular solution has been shown to selectively increase the frequency of mEPSCs in numerous preparations (e.g. Fatt and Katz, 1952; Bekkers and Stevens, 1989). Increasing the osmolarity of the aCSF with 50 mM sucrose resulted in a shift in the mEPSC interval distribution to shorter intervals (Fig. 3-10A, $P < 0.0001$, K-S test) without a coincident change in the amplitude distribution (Fig. 3-10B, $P > 0.19$, K-S test, $N = 3$).

Figure 3-8. NPY does not affect mEPSC amplitude or interval distributions from a CA3 pyramidal neuron treated with cadmium

A. Cumulative probability plot of the mEPSC amplitude distribution from a CA3 pyramidal neuron which was treated with 200 μM Cd^{2+} instead of 1 μM TTX. NPY does not alter the mEPSC amplitude distribution ($P > 0.05$, K-S test). **B**. Cumulative probability plot of the mEPSC interval distribution from the same cell in A. NPY (1 μM) does not alter the mEPSC interval distribution ($P > 0.61$, K-S test).


A
Cumulative Probability
1.0
0.5
0.0
Control
1μM NPY
0
-100
-200
-300
mEPSC Amplitude (pA)

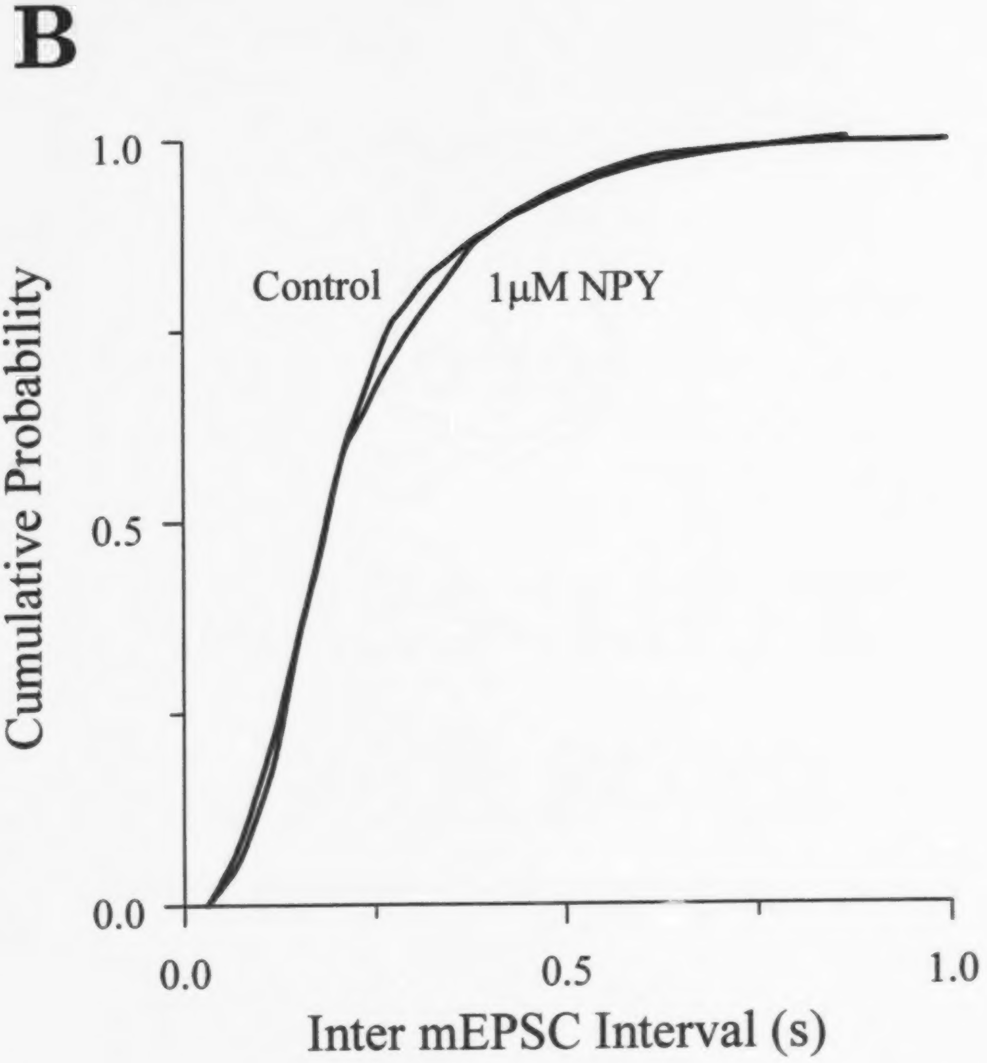
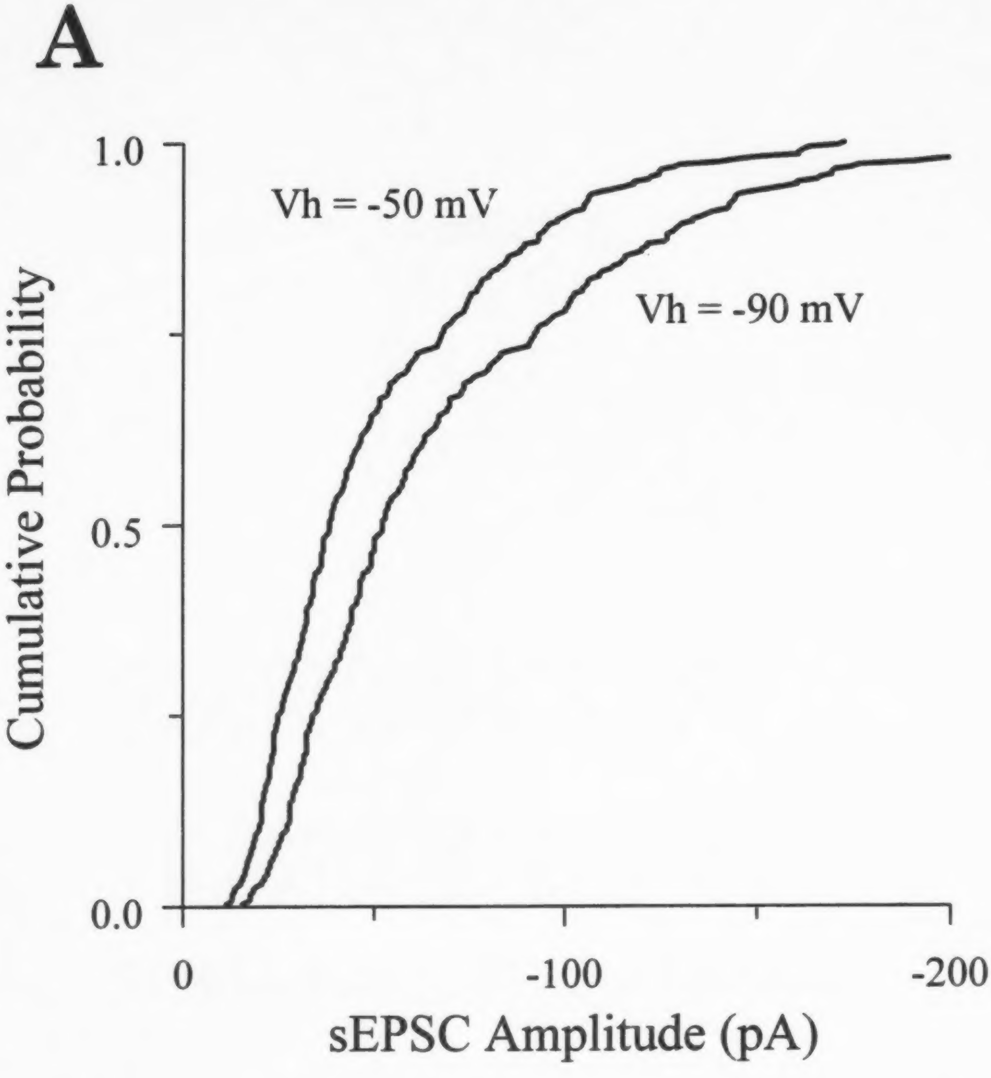

B
Cumulative Probability
Control
1μM NPY
1.0
0.5
0.0
0.0
0.5
1.0
Inter mEPSC Interval (s)

Figure 3-9. Changes in neuron holding potential alters the sEPSC amplitude but not interval, distribution of a CA3 pyramidal neuron

A. Cumulative probability plot of the sEPSC amplitude distribution. A more negative holding potential (V_m = -90 mV) shifts the sEPSC amplitude distribution to larger amplitudes compared to more depolarized holding potentials (V_m = -50 mV) ($P < 0.0001$, K-S test). **B**. Cumulative probability plot of the sEPSC interval distribution. Holding potential does not alter the sEPSC interval distribution ($P > 0.33$, K-S test).


A
1.0
0.5
0.0
Cumulative Probability
Vh = -50 mV
Vh = -90 mV
0
-100
-200
sEPSC Amplitude (pA)

B

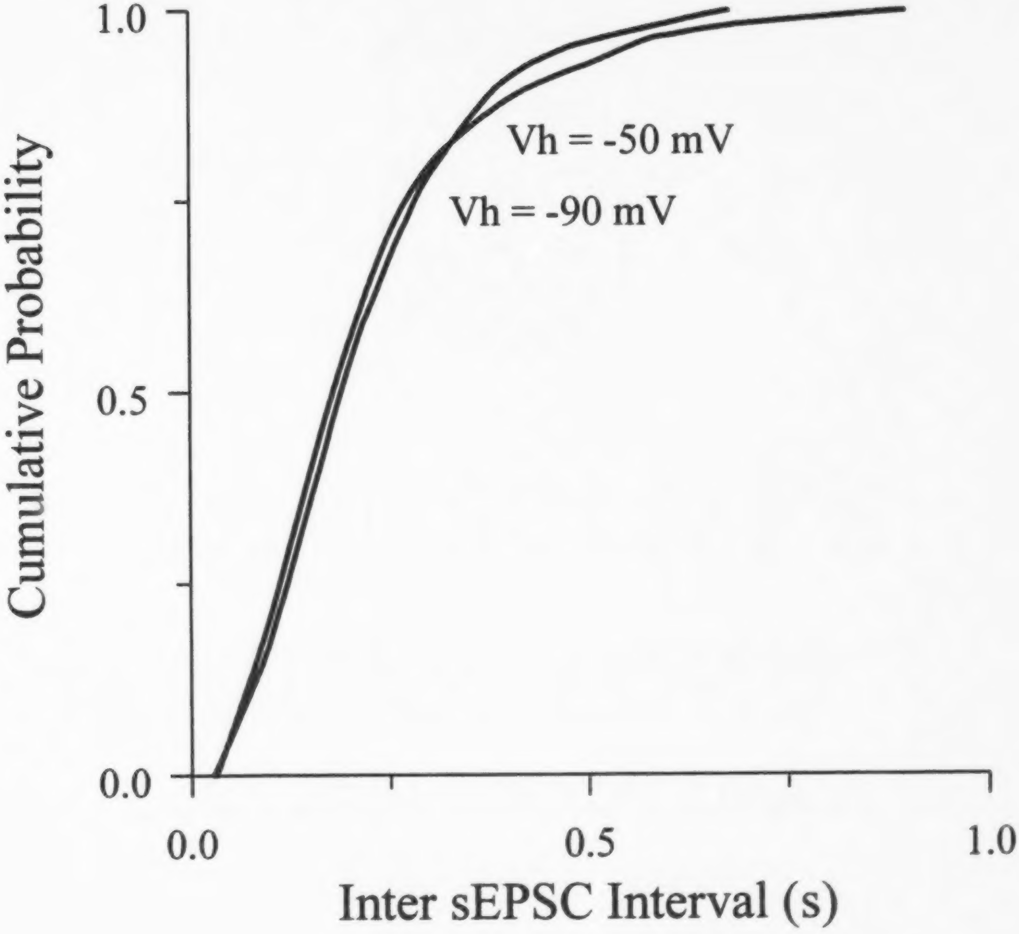
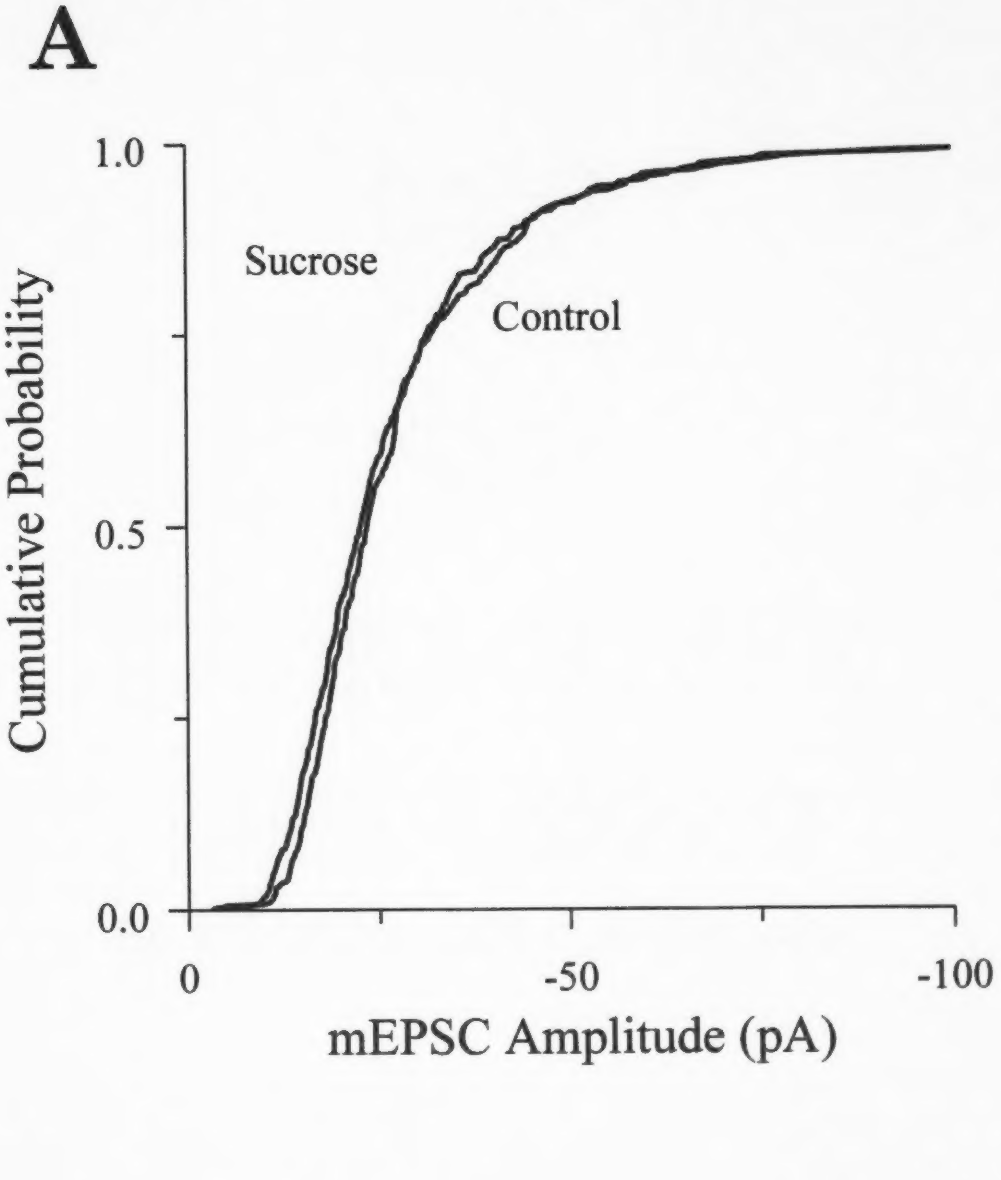
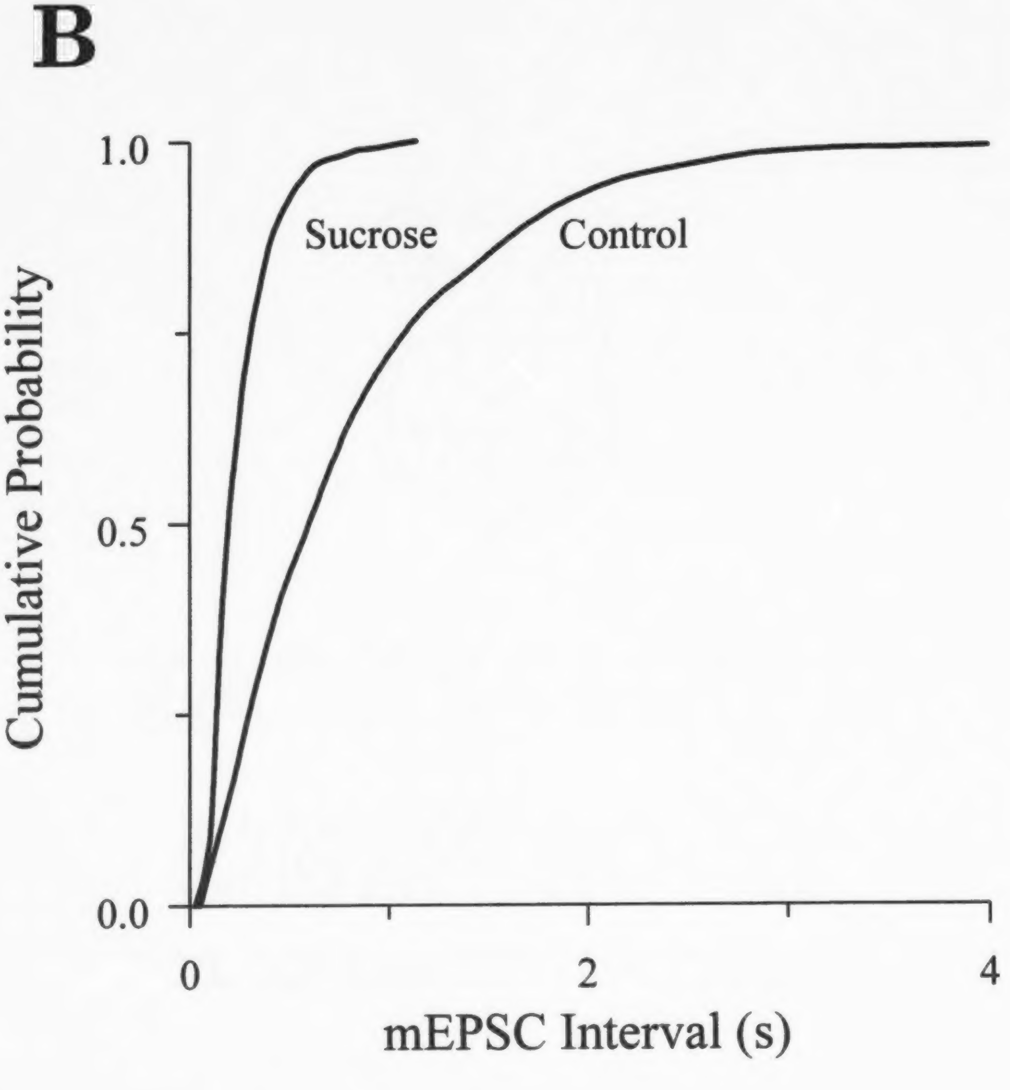

1.0
0.5
0.0
Cumulative Probability
Vh = -50 mV
Vh = -90 mV
0.0
0.5
1.0
Inter sEPSC Interval (s)

Figure 3-10. Sucrose alters the mEPSC interval, but not amplitude, distribution of a CA3 pyramidal neuron

A. Cumulative probability plot of the mEPSC amplitude distribution. Sucrose (50 μM) does not alter the mEPSC amplitude distribution ($P > 0.19$, K-S test). **B**. Cumulative probability plot of the mEPSC interval distribution. Sucrose shifts the mEPSC interval distribution to smaller intervals ($P < 0.0001$, K-S test).


A
1.0
0.5
0.0
Cumulative Probability
Sucrose
Control
0
-50
-100
mEPSC Amplitude (pA)


B
1.0
0.5
0.0
Cumulative Probability
Sucrose
Control
0
2
4
mEPSC Interval (s)

Discussion

The data from the present study demonstrate that NPY acts presynaptically to inhibit excitatory synaptic transmitter release onto CA3 pyramidal neurons from acutely prepared rat hippocampal slices. NPY appears to activate a Y_2 receptor and acts through a mechanism that modulates only activity-dependent transmitter release.

We believe we have accurately measured pre- and postsynaptic changes for the following reasons. First, treatment of the slice with sucrose to increase extracellular osmolarity, a manipulation which increases spontaneous release with no postsynaptic activity (Fatt and Katz, 1952; Bekkers and Steven, 1989), results in a selective decrease in sEPSC or mEPSC intervals with no effect on their amplitudes. This is consistent with a purely presynaptic modification. Second, changing the membrane potential of the cell alters the driving force for the sEPSCs and mEPSCs through the postsynaptic receptor, which should selectively alter the postsynaptic (quantal EPSC) amplitudes, without affecting the presynaptic release (intervals). This manipulation results in the detection of a change in quantal EPSC amplitudes without a change in their intervals. This is consistent with a purely postsynaptic manipulation. Therefore, selective pre- or postsynaptic manipulations can be accurately detected by our experimental protocol.

Site of action of NPY

Our results demonstrate that NPY acts to inhibit the release of glutamate from the presynaptic terminals onto CA3 pyramidal neurons of the rat hippocampal slice. This conclusion is based on the observation that NPY or [ahx$^{5-24}$]NPY affects neither the amplitudes nor the intervals of mEPSCs in any of the cells tested. Because NPY did not affect mEPSC amplitudes, we conclude that NPY does not act postsynaptically to alter excitatory synaptic transmission. Furthermore, because TTX or Cd^{2+} eliminates the effect of NPY and [ahx$^{5-24}$]NPY on the interval distribution of spontaneous transmitter release, NPY can affect only those events which require presynaptic terminal activity.

The initial observation that NPY increased the interval between successive sEPSCs but also reduced the amplitude of sEPSCs in 69 % of cells tested could be interpreted to support the conclusion that NPY acts both pre- and postsynaptically to inhibit excitatory synaptic transmission, as occurs with $GABA_B$ and adenosine A_1 receptor activation (for review see Nicoll *et al.*, 1990). However, in 31 % of cells, NPY shifted the interval distribution of sEPSCs to shorter intervals without a coincident change in the amplitude distribution. Therefore, in this population of cells, NPY acts in a manner consistent with a presynaptic mechanism.

In some cells, NPY receptor modulation of both sEPSCs and mEPSCs was studied. In the majority of these cells, NPY caused a shift in the sEPSC amplitude distribution to smaller events. Application of TTX to these cells to isolate mEPSCs should only eliminate Na^+-dependent action potentials and consequently affect have only a presynaptic action. However, TTX not only caused a shift in the interval distribution (as expected for presynaptic change) but also shifted the amplitude distribution. In addition, both [ahx$^{5-24}$]NPY and TTX reduced the relative frequency of larger amplitude sEPSCs compared to control sEPSC amplitudes. It is unlikely that TTX has a postsynaptic effect upon quantal EPSCs. Furthermore, NPY did not change the sEPSC amplitude distribution in 31 % of cells. We have therefore interpreted these observations to be the result of NPY and TTX preferentially inhibiting larger sEPSCs. It is possible such larger events may arise from the temporal or spatial summation of smaller events, perhaps due to the higher probability of summation of events as a result of the higher frequency of sEPSCs (Ankri *et al.* 1994). Similar observations and interpretations have been made for studies on μ-opioid receptor inhibition of sIPSCs in the rat hippocampus (Cohen *et al.* 1992; Rekling, 1993; Lupica, 1995). Application of μ-receptor agonists inhibits both the amplitude and frequency of sISPCs, but has no effect on mIPSC amplitudes. TTX also causes a shift in the amplitude distribution of sIPSCs (Lupica, 1995). These observations were interpreted to result from the selective inhibition of large sISPCs (Cohen *et al.* 1992; Lupica, 1995). The present experiments cannot exclude the possibility that some of the larger events result from an interaction of single or multiple quantal synaptic potentials with voltage-dependent Na$^+$ or Ca^{2+} conductances on the dendrites of the postsynaptic cells (Magee and Johnston, 1995). If this were to occur, then the amplitudes of some synaptic events would also be expected to distort the waveform of summating, smaller quantal events to appear more like one large event. This mechanism could potentially explain the relative loss of larger events in the presence of TTX or NPY. This hypothesis could be tested in experiments where postsynaptic Na$^+$ conductances are blocked by addition of QX-314 or the like to the pipette.

Mechanism of action of NPY

, Both NPY and [ahx$^{5-24}$]NPY produce similar effects on sEPSCs recorded in CA3 pyramidal neurons. The activity of [ahx$^{5-24}$]NPY, a Y$_2$ receptor selective agonist (Beck-Sickinger *et al.* 1992), suggests that NPY activates a Y$_2$ receptor to inhibit excitatory synaptic transmission onto CA3 pyramidal cells. These findings are in agreement with receptor binding studies, which show a preponderance of Y$_2$ receptors in strata radiatum and oriens of regions CA1 and CA3 of the rat hippocampus (Dumont, Fournier, St-

Pierre, and Quirion, 1993). Furthermore, studies in CA1 have shown the NPY inhibition of excitatory synaptic transmission to be due to the activation of Y_2 receptors (Colmers *et al.* 1991).

NPY and [ahx$^{5-24}$]NPY do not affect mEPSC amplitude or interval distributions. This lack of effect suggests that action potentials, and hence presynaptic activity, is required for NPY to exert its action. This is supported by the observation that NPY has no effect on the mEPSCs observed in the presence of Cd^{2+} alone. Together, these data suggest that TTX (1 μM) entirely eliminates Ca^{2+} influx-dependent transmitter release and that the Y_2 receptor most likely modulates the influx of Ca^{2+}, either directly or indirectly. Similar findings have been seen in CA3 pyramidal cells in organotypical slice cultures, where α_1 adrenoceptor activation presynaptically inhibits excitatory synaptic transmission without affecting the amplitude or frequency of mEPSCs (Scanziani *et al.* 1993). In contrast, both $GABA_B$ and adenosine A_1 receptor activation inhibits mEPSC frequency without affecting mEPSC amplitudes in CA3 pyramidal neurons of these cultures. A_1 receptors inhibit mEPSC frequencies in hippocampal neurons in primary culture (Scanziani *et al.* 1992; Scholz and Miller, 1992). This suggests that some presynaptic receptors inhibit excitatory transmitter release at least in part by an action to the influx of Ca^{2+} (Silinsky, 1984). However, the lack of an effect by NPY on mEPSC amplitude and interval distributions strongly supports the conclusion that the regulation of Ca^{2+} influx is the predominant action of the Y_2 receptor.

The precise mechanism of action of NPY at the presynaptic terminal cannot be determined from the present experiments. Our results are consistent either with the activation or potentiation of presynaptic K^+ currents, or the direct inhibition of presynaptic voltage-dependent Ca^{2+} channels (VDCC).

NPY also modulates the release of transmitter from other neurons and cell types. In dorsal root ganglion cells in culture (DRG), NPY has been shown to inhibit VDCCs in the cell bodies and inhibit the release of substance P and acetylcholine (Walker, Ewald, Perney and Miller, 1988). NPY inhibits VDCCs but also activates inwardly rectifying K^+ channels of frog sympathetic neurons (Schofield and Ikeda, 1988; Zidichouski, Chen and Smith, 1990). NPY modulates Na^+ channels, K^+ channels and VDCCs in frog melanotrophs (Valentijn, Vaudry, Kloas and Cazin, 1994). More importantly, studies of rat sympathetic neurons co-cultured with atrial myocytes have shown that Y_2 receptor activation inhibits transmitter release by directly inhibiting N-type Ca^{2+} channels at the presynaptic terminal (Toth, Bindokas, Bleakman, Colmers and Miller, 1993).

In the rat hippocampus, NPY does not affect the passive membrane properties of pyramidal neurons of CA1 or CA3 or granule cells of the dentate gyrus (Klapstein and Colmers, 1993) suggesting NPY is not modulating resting K^+ channels there. Recently, however, we have shown that NPY inhibits N-type Ca^{2+} channels in rat dentate granule cells (McQuiston *et al.* 1994). If action at the cell body is any indication of what may happen at the terminal, as occurs in rat sympathetic synapses, then NPY may directly inhibit the activation of VDCCs at the terminal of CA3 excitatory synapses. This could be tested using experiments similar to those of Toth and colleagues (1993). With recent development of methods to selectively load presynaptic terminals of acutely prepared slices with Ca^{2+}-sensitive dyes (Regehr and Tank, 1991; Wu and Saggau, 1994), the hypothesis that NPY inhibits Ca^{2+} channels of the presynaptic terminal on CA3 pyramidal neurons inhibited by NPY might be tested directly.

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Chapter 4

Neuropeptide Y_1 receptors inhibit N-type calcium currents and reduce transient calcium increases in rat dentate granule cells

Neuropeptide Y (NPY) and its receptors are found in large amounts in widely-distributed regions of the vertebrate peripheral (PNS) and central nervous system (CNS) (Dumont *et al.*, 1990, 1992, 1993; Hendry , 1993; Sundler *et al.*, 1993; Wahlestedt and Reis, 1993). Furthermore, NPY has been shown to have a variety of physiological, behavioral and autonomic functions in both the mammalian PNS and CNS (Colmers and Bleakman, 1994; Wahlestedt and Reis, 1993). However, the cellular mechanisms of NPY's actions are less well understood.

In the mammalian CNS, NPY and its receptors are found in perhaps the highest concentrations in the hippocampus (Dumont *et al.*, 1992, 1993; Hendry , 1993). NPY binding is found in high density in the strata radiatum and oriens of the hippocampus proper, where NPY potently and selectively inhibits excitatory synaptic transmission onto CA1 (Colmers *et al.*, 1987, 1988; Haas *et al.*, 1987) and CA3 pyramidal neurons (McQuiston and Colmers, 1992; Klapstein and Colmers, 1993) via a Y_2 subtype receptor. In the hippocampal formation of the rat, NPY is mostly concentrated in the dentate gyrus (Hendry, 1993) and substantial specific binding for NPY has been shown to be present in the molecular layer of the dentate gyrus (Dumont *et al.*, 1990, 1993) where afferent inputs from the entorhinal cortex synapse onto dentate granule cells (DGC) (Laatch and Cowan, 1967; Hjorth-Simonsen and Jeune, 1972). The receptor in the dentate gyrus is mostly Y_1, in contrast to areas CA1 and CA3 (Dumont et al., 1990, 1992, 1993; Aicher *et al.*, 1991; Wahlestedt and Reis, 1993). However, no effect of NPY has been observed on synaptic transmission onto DGC's or on the passive electrical properties of the DGC's (Klapstein and Colmers, 1993) nor does NPY affect somatic properties of other hippocampal neurons (Colmers *et al.*, 1988; Bleakman *et al.*, 1992). Therefore, a function for NPY and its receptors in the dentate gyrus remains elusive.

In the PNS, NPY has been shown to inhibit N-type voltage-dependent calcium channels (VDCC's) of vertebrate sympathetic neurons, rat myenteric plexus neurons, rat sensory neurons (for review see Bleakman *et al.*, 1993; Colmers and Bleakman, 1994). NPY also inhibits both N and L-type VDCC's of frog melanotrophs (Valentijn *et al.*, 1994). In most cases, NPY inhibits the N-type VDCC's by activating a Y_2 receptor. By contrast, in a subpopulation of rat nodose ganglion neurons, the Y_1 receptor potentiates these same VDCC's (Wiley *et al.*, 1993). NPY has also been shown to augment VDCC's in rat vascular smooth muscle (Xiong *et al.*, 1993). Furthermore, NPY has often been shown to be coupled to intracellular calcium mobilization in neurons and other cell types (Michel, 1991; Dumont *et al.*, 1992; Wahlestedt and Reis, 1993).

Therefore we investigated the possibility that NPY acts to either modulate intracellular calcium homeostasis or modulate VDCC's of rat hippocampal DGC's. Here we show that NPY inhibits N-type VDCCs and reduces activity dependent Ca^{2+} increases in rat hippocampal DGCs predominantly by activating a Y_1 receptor.

Materials and Methods

Calcium Imaging Experiments

Experiments were performed on dentate granule cells (DGCs) in 450 μm transverse slices of 21 to 30 day old male Sprague Dawley rats. Rats were anaesthetized with halothane and decapitated. Brains were rapidly removed and placed in ice cold saline (4°C). The hippocampus was then dissected from the brain and cut on a vibrotome. Slices were placed on a polypropylene mesh, submerged and continuously perfused with saline (23°C) containing (in mM) NaCl 124, KCl 2, $MgSO_4$ 1.3, KH_2PO_4 1.25, $NaHCO_3$ 18, $CaCl_2$ 2.5, glucose 10 equilibrated with 95% O_2/ 5% CO_2 (pH 7.4). Recordings from DGCs were obtained in the whole cell patch clamp configuration (Blanton *et al.*, 1989) using patch electrodes (5 to 9 MΩ) filled with 200 microM fura-2 (K^+ salt; Molecular Probes, Eugene, OR) dissolved in the intracellular solution consisting or (in mM) K Gluconate 135, MgATP 2, NaGTP 0.3, HEPES 10 (pH 7.2). Neurons were voltage clamped at a holding potential of -60 mV in the continuous voltage clamp mode using an Axoclamp 2A amplifier and 500 ms voltage steps to 0 mV were made periodically in order to stimulate voltage-dependent Ca^{2+} influx.

Imaging experiments were performed from the top surface of the slice using an upright microscope (Zeiss Axioscope) and a 40x water immersion objective (Zeiss). Digital images were taken with a cooled CCD camera system (Photometrics LTD, Tuscon, AR) used in the frame-transfer mode to acquire image pairs at 360 and 380 nm excitation wavelengths. Ca^{2+} levels were calculated from background-corrected images using the ratio method (Grynkiewicz *et al.*, 1985; Tsien, 1989). Calibration of the indicator was carried out under *in vitro* conditions using general procedures described previously (Connor, 1986). For these measurements, a light path of 20 μm was achieved by confining the indicator-saline mixtures in a 20 x 50 μm capillary (Vitro Dynamics, Rockway, NJ).

Dissociated Cell Preparation

Single DGC neurons were isolated from 21 to 30 day old male Sprague-Dawley rats by a previously described procedure (Kay, 1989; White *et al.*, 1993). Rats were anaesthetized with halothane and decapitated. Brains were rapidly removed and placed in ice cold (4°C) bicarbonate based artificial cerebral spinal fluid (aCSF) saturated with 95% O_2/5% CO_2. The brain was hemisected sagitally by a razor blade and cut on a vibrotome into 500 μm transverse slices containing the hippocampus. The dentate gyrus was cut out and placed in a spinner flask (Belco) containing warm (30°C) dissociation solution saturated with 100% O_2. Slices were allowed approximately 15 min to recover and subsequently treated with 0.2 mg/ml proteinase K (Sigma). After 5 min the solution was exchanged for another solution containing 1mg/ml trypsin type XI (Sigma) and treated as such for a further 30 min. Following trypsin treatment the slices were removed, placed in dissociation solution and allowed to return to room temperature. The slices were allowed 1 to 2 hours to recover. Following recovery, two slices were triturated in dissociation solution (to which 0.5 mg/ml bovine serum albumin was added) through two fire-polished pasteur pipettes with tip diameters of approximately 500 μm and 200 μm respectively. Cells were placed in a recording chamber and allowed to settle onto polylysine coated glass cover slips for 10 to 15 min before washing with a HEPES based aCSF.

Calcium Current Recording

Currents were recorded from DGC neurons at room temperature (approx. 22°C) using the whole-cell patch clamp configuration (Hamill *et al.*, 1981). with an axopatch 1D amplifier and analyzed with pClamp software (Axon instruments). Patch electrodes made from borosilicate glass (World Precision Instruments) were not fire-polished or insulated. Whole cell recording was obtained in the HEPES-buffered aCSF, which was then promptly switched to the calcium current solution. Series resistance and membrane capacitance were electronically compensated by 80 to 90 % via the amplifier circuitry and were continuously monitored throughout the experiment. Currents were leak subtracted using a P/N protocol and filtered at 1 kHz. Currents were generated with 40 ms voltage steps (at 20 s intervals) to +5 mV from a holding potential of -60 mV or voltage ramps from -80 mV to +70 mV over 100 ms. DGC neurons were superfused with solutions in a chamber mounted on an inverted microscope (Nikon). Drugs were applied by bath superfusion.

Significant differences in mean values were tested for using the Wilcoxon signed-ranks test or the Kruskal-Wallis test. Values were considered significantly differences for P values less than 0.05.

Solutions and Materials

For the dissociated cell experiments, the dissociation solution consisted of (in mM) NaCl 115, KCl 5, PIPES 20, $CaCl_2$ 1, $MgCl_2$ 4, glucose 25, kynurenic acid 1, Na-pyruvate 1. The bicarbonate-buffered aCSF used for the dissection consisted of (in mM) NaCl 126, KCl 2.5, $MgCl_2$ 6, $CaCl_2$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26, glucose 10, kynurenic acid 1. The HEPES-buffered solution used to wash the isolated cells consisted of (in mM) NaCl 135, KCl 2.5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 20, glucose 20, Na-pyruvate 1. The calcium current solution consisted of (in mM) NaCl 100, KCl 2.5, $MgCl_2$ 2, $CaCl_2$ 5, HEPES 20, CsCl 3, tetraethylammonium chloride (TEA Cl) 30, 4-aminopyridine 5, glucose 10, Na-pyruvate 1. All extracellular solutions had a pH of 7.4 and an osmolarity of 315 to 320 mOsm. The intracellular electrode solution consisted of (in mM) CsCl 100, TEA Cl 30, HEPES 10, MgATP 4, Na_2GTP 0.3, $BAPTAK_4$ 0.1, ditris phosphocreatine 14, creatine phosphokinase 50 units/ml pH 7.2 (with CsOH) and 280 to 290 mOsm. For the barium current experiments, the calcium of the extracellular calcium current solution was replaced by equimolar barium and the $BAPTAK_4$ of the intracellular electrode solution was replaced by BAPTA free acid (20mM). The CsCl concentration was reduced to compensate for the increased BAPTA concentration.

NPY was obtained from Richelieu Biotechnology Inc. (Quebec), [$Leu^{31}Pro^{34}$]-NPY, $NPY_{13\text{-}36}$ and PYY were obtained from Bachem, ωCTX-GVIA was obtained from Peninsula Laboratories, Nimodipine was a generous gift from Miles, Inc., baclofen (Lioresal) was a gift of Ciba-Geigy, and creatine phosphokinase was obtained from Boehringer Mannheim. All other compounds were obtained from SIGMA Chemical Company.

Results

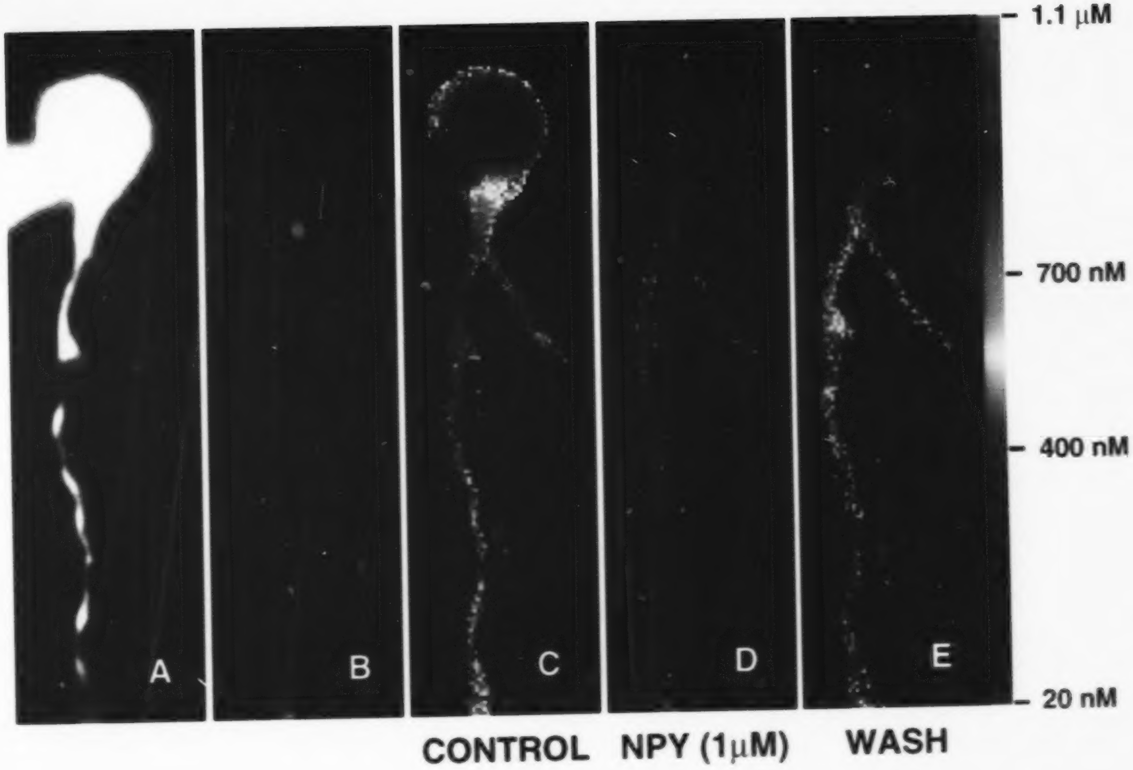
NPY Inhibits Depolarization-induced Increases in [Ca2+]i of DGCs

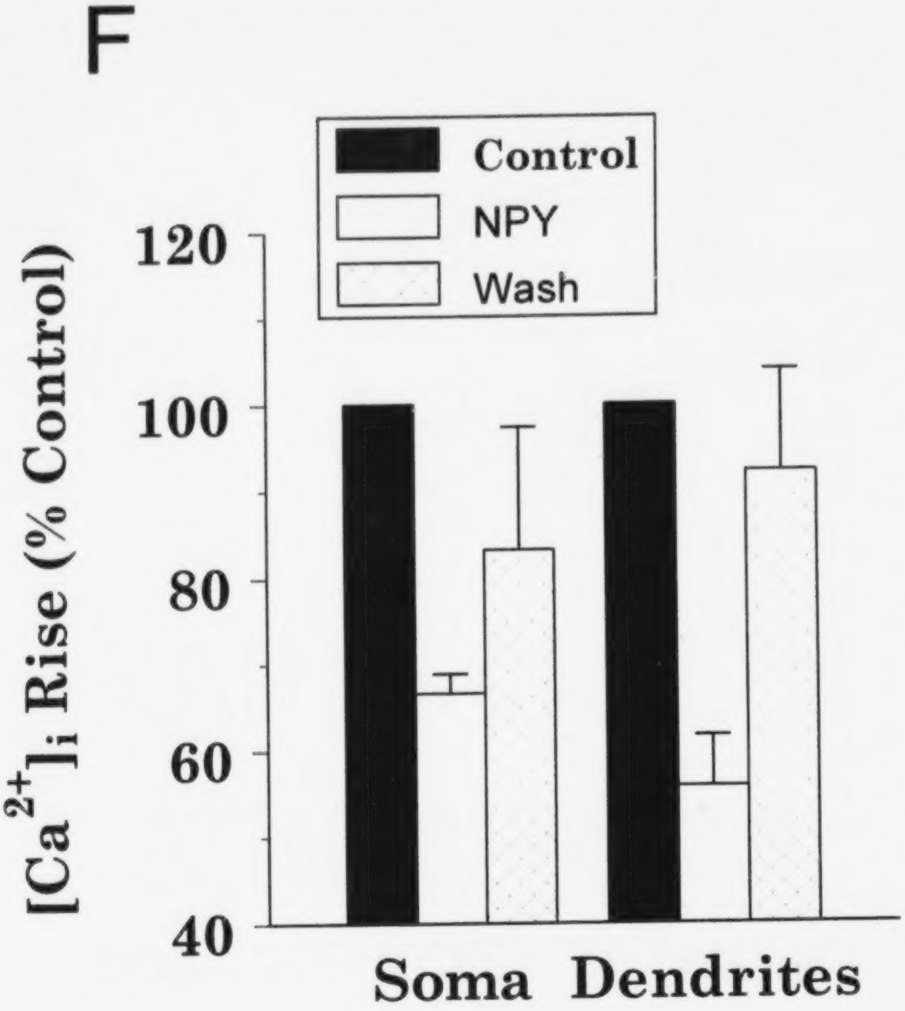
We first sought to determine if NPY modulated either resting $[Ca^{2+}]_i$ or Ca^{2+} influx in DGCs. In order to do this, we made spatially-resolved measurements of $[Ca^{2+}]_i$ in DGC's, loaded with fura-2 (Grynkewicz et al., 1985; see methods) via a whole-cell patch electrode that also served to measure voltage and injected current. An experiment showing the effects of NPY is illustrated in Figure 4-1. The resting $[Ca^{2+}]_i$ was not altered by bath application of 1 μM NPY. In order to investigate a possible NPY modulation of Ca^{2+} influx, we depolarized the soma from -60 mV to 0 mV for 500 ms via the patch pipette. No attempts were made to isolate calcium currents (I_{Ca}) and the depolarization produced Na^+- and Ca^{2+}-dependent action potentials which we did not attempt to control. NPY (1 μM) significantly decreased the depolarization-induced rise in $[Ca^{2+}]_i$ compared to controls (Fig. 4-1c,d). Washout of NPY partially reversed the decrease (Fig. 4-1e). The data from 5 experiments are summarized in the histogram (Fig. 4-1f). NPY (1 μM) significantly decreased the depolarization-induced rise in $[Ca^{2+}]_i$ to 66.6 ± 2.3% of the control rise in the soma (Fig. 4-1f) and to 55.8% ± 5.9% of the control rise in the dendrite (Fig. 4-1f, $P < 0.05$, Wilcoxon signed-ranks test). The larger decrease of $[Ca^{2+}]_i$ in the dendrites compared to the soma was not significantly different ($P > 0.05$, Wilcoxon signed-ranks test).

The most likely means by which NPY could inhibit this depolarization-induced increase in $[Ca^{2+}]_i$ is by 1) inhibiting VDCCs directly or 2) reducing the excitability of the DGC, as by increasing K^+ channel activity or decreasing Na^+ channel activity, and thereby the influx of Ca^{2+}. We first tested the hypothesis that NPY inhibited VDCCs of

Figure 4-1. NPY Inhibits Depolarization-induced Increases in $[Ca^{2+}]_i$ in DGCs from Hippocampal Slices.

Top Panel: Image of a DGC in a hippocampal slice of the rat. A. Fluorescence image with the neuron's cell body at the top and a bifurcating dendrite underneath. The $[Ca^{2+}]_i$ coding for the false color image is on the far right of the figure. The cell was voltage clamped at -60 mV and periodically depolarized to 0 mV for 500 ms. B. Ratiometric image of the $[Ca^{2+}]_i$ of the cell at rest. C. The control rise in $[Ca^{2+}]_i$ in response to a depolarizing step. D. The presence of NPY (1 μM) inhibits the rise in $[Ca^{2+}]_i$ following a depolarizing step. E. The rise in $[Ca^{2+}]_i$ following a depolarizing step recovers toward control levels after washing NPY. Bottom Panel: F. Histogram summarising results of similar experiments (n = 5). NPY (1 μM) significantly ($P < 0.05$, Wilcoxon signed-ranks test) inhibited depolarization-induced rises in $[Ca^{2+}]_i$ of dendrites in the molecular layer and soma of DGCs of rat hippocampal slices.


A
B
C
D
E
CONTROL
NPY (1μM)
WASH
1.1 μM
700 nM
400 nM
20 nM


F
Control
NPY
Wash
$[Ca^{2+}]_i$ Rise (% Control)
120
100
80
60
40
Soma
Dendrites

DGCs.

NPY Inhibits Voltage-dependent Calcium Currents (I_{Ca}) of Acutely Dissociated DGCs

We acutely isolated DGCs from rat hippocampal slices and pharmacologically isolated I_{Ca} as described in Methods. DGCs were distinguished from other neuronal types by their small round cell bodies (Mody *et al.*, 1989). Larger DGCs were selected because they usually had a larger I_{Ca}, which also ran down more slowly and recovered more completely from NPY inhibition. DGCs with larger dendrites were also selected because NPY binding sites are concentrated in their dendrites (Dumont *et al.*, 1990, 1993; Aicher *et al.*, 1991). Unless otherwise indicated, I_{Ca}'s from DGCs were recorded with 5 mM Ca^{2+} in the extracellular solution as the charge carrier and with a pipette solution having low Ca^{2+} buffering (0.1 mM BAPTA with no added Ca^{2+}, see methods).

I_{Ca} of these DGCs were typically between 200 pA to 1000 pA in peak amplitude. The maximum peak amplitudes were elicited with voltage steps from -60 mV to between +5 mV and +10 mV. The I_{Ca} of 42 of 50 acutely dissociated DGCs was inhibited by 1 μM NPY (Fig. 4-2). Not all cells recovered fully from NPY inhibition, and recovery in many cells took several minutes longer than was necessary for complete exchange of the solution in the recording chamber. Some cells showed no recovery within the recording period. Recovery of I_{Ca} from NPY inhibition was also obscured by the rapid rate of I_{Ca} rundown despite the presence of an ATP-regenerating solution (see methods). Repeated administration of NPY often resulted in a loss of NPY's ability to inhibit I_{Ca}.

NPY produced two types of inhibition of I_{Ca} in DGCs. First, in the great majority of DGCs, NPY caused a reversible inhibition of I_{Ca} without an alteration in the timecourse of I_{Ca} activation (Fig. 4-2a). The second type of I_{Ca} inhibition by NPY in DGCs was accompanied by a slowing of the activation timecourse of I_{Ca} (Fig. 4-2b). This second type of inhibition was seen less frequently, however, it was often more robust and reversible than the other (Figs. 4-3b). Peak I_{Ca} in DGCs which showed a change in I_{Ca} timecourse following NPY application was inhibited by 38.8% ± 5.4% (in 12 of 45 DGCs), as opposed to the 22.3% ± 0.9% (in 33 of 45 DGCs; $P < 0.001$, Kruskal-Wallis) inhibition of peak I_{Ca} observed in DGCs with no accompanying change in I_{Ca} activation timecourse.

NPY Inhibits I_{Ca} of DGCs Predominantly by Activating a Y_1 receptor.

At present, NPY appears to have 3 receptor subtypes, termed Y_1, Y_2 and Y_3 (Grundemar *et al.*, 1993; Wahlestedt and Reis, 1993). We tested the receptor profile for NPY using [Leu^{31}Pro^{34}]-NPY (Y_1-selective), NPY13-36 (Y_2-selective) and PYY (Y_1 and Y_2 but not Y_3-selective), in addition to the nonselective agonist, NPY itself.

One such experiment is illustrated in Figure 4-3. The top panel of Figure 4-3 (a) is a time plot of an experiment in which NPY and the receptor selective agonists were applied to the same cell. NPY, [Leu$_{31}$Pro$_{34}$]-NPY and PYY significantly inhibited the peak I_{Ca}, while NPY_{13-36} did not (Fig. 4-3a). In this case, since NPY_{13-36} can activate both Y_2 and Y_3 receptors but was inactive here, NPY must inhibit the I_{Ca} of this DGC via a Y_1 receptor. The current traces show that NPY, [Leu$_{31}$Pro$_{34}$]-NPY and PYY slow the activation timecourse of the I_{Ca} (Fig. 4-3b).

Although most DGCs appear to express only Y_1 receptors (Fig. 4-3), other DGCs express more than one NPY receptor subtype (Fig. 4-4). In the DGC illustrated in Figure 4-4, NPY and all 3 selective agonists inhibited the I_{Ca}. This experiment shows that more

Figure 4-2. NPY Inhibits I_{Ca} of Acutely Dissociated DGCs.

NPY inhibits the I_{Ca} of two different DGCs. The I_{Ca} was elicited by a 40 ms voltage step to +5 mV from a holding potential of -60 mV. Voltage steps were elicited at 20 s intervals. A. 1 μM NPY inhibits the I_{Ca} of this DGC without altering the timecourse of activation of the current. B. In a different DGC, NPY (1 μM) inhibits I_{Ca} and slows the timecourse of activation of the current.

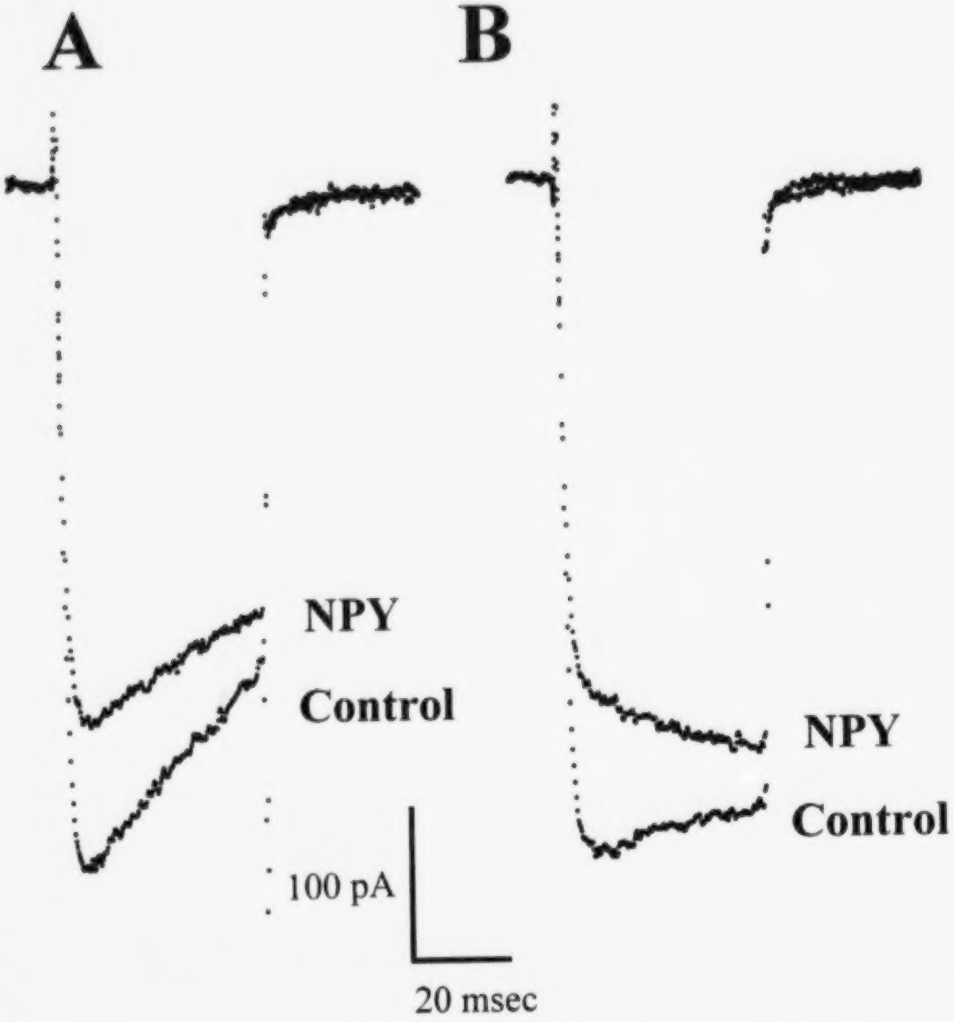
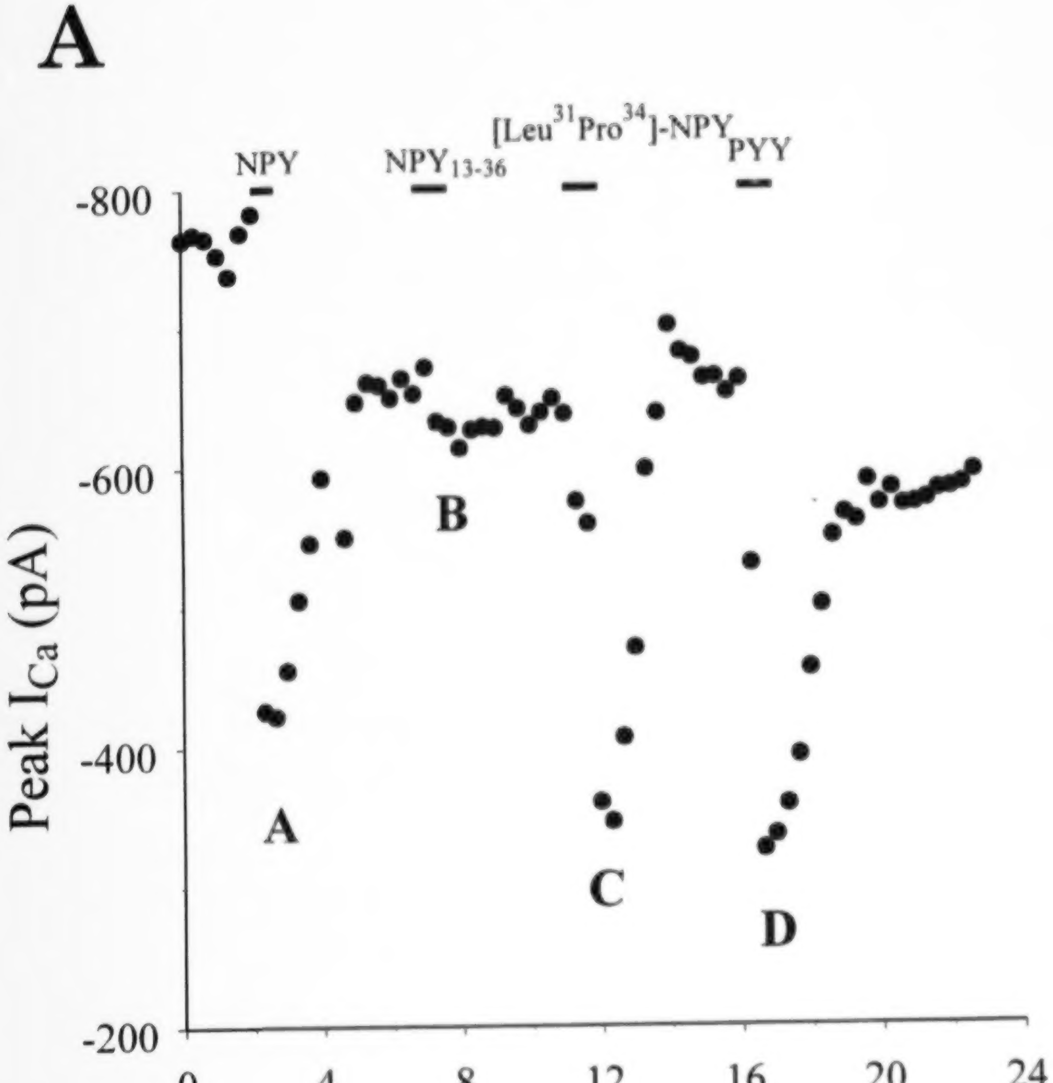

A
B
NPY
Control
NPY
Control
100 pA
20 msec

Figure 4-3. NPY Inhibits I_{Ca} of an Acutely Dissociated DGC via a Y_1 receptor.

Top panel. Time plot of peak I_{Ca} from a DGC elicited at 20 s intervals by a 40 ms voltage step to +5 mV from a holding potential of -60 mV. The peak current elicited was plotted against the time elapsed from the beginning of the experiment. Drugs were applied during the times indicated by the bars at the top of the plot. NPY, [$Leu^{31}Pro^{34}$]-NPY and PYY inhibited the I_{Ca} but NPY_{13-36} did not. All drug concentrations were 1 μM. Bottom panel. The I_{Ca} elicited at the times indicated by the corresponding letters in the time plot of the top panel. The control traces were taken immediately before the respective drug application. Horizontal scale bar: 20 ms, vertical scale bar: 300 pA.


A
NPY
NPY13-36
[Leu31Pro34]-NPY
PYY
-800
-600
-400
-200
Peak ICa (pA)
A
B
C
D
0
4
8
12
16
20
24
Time (min)

B

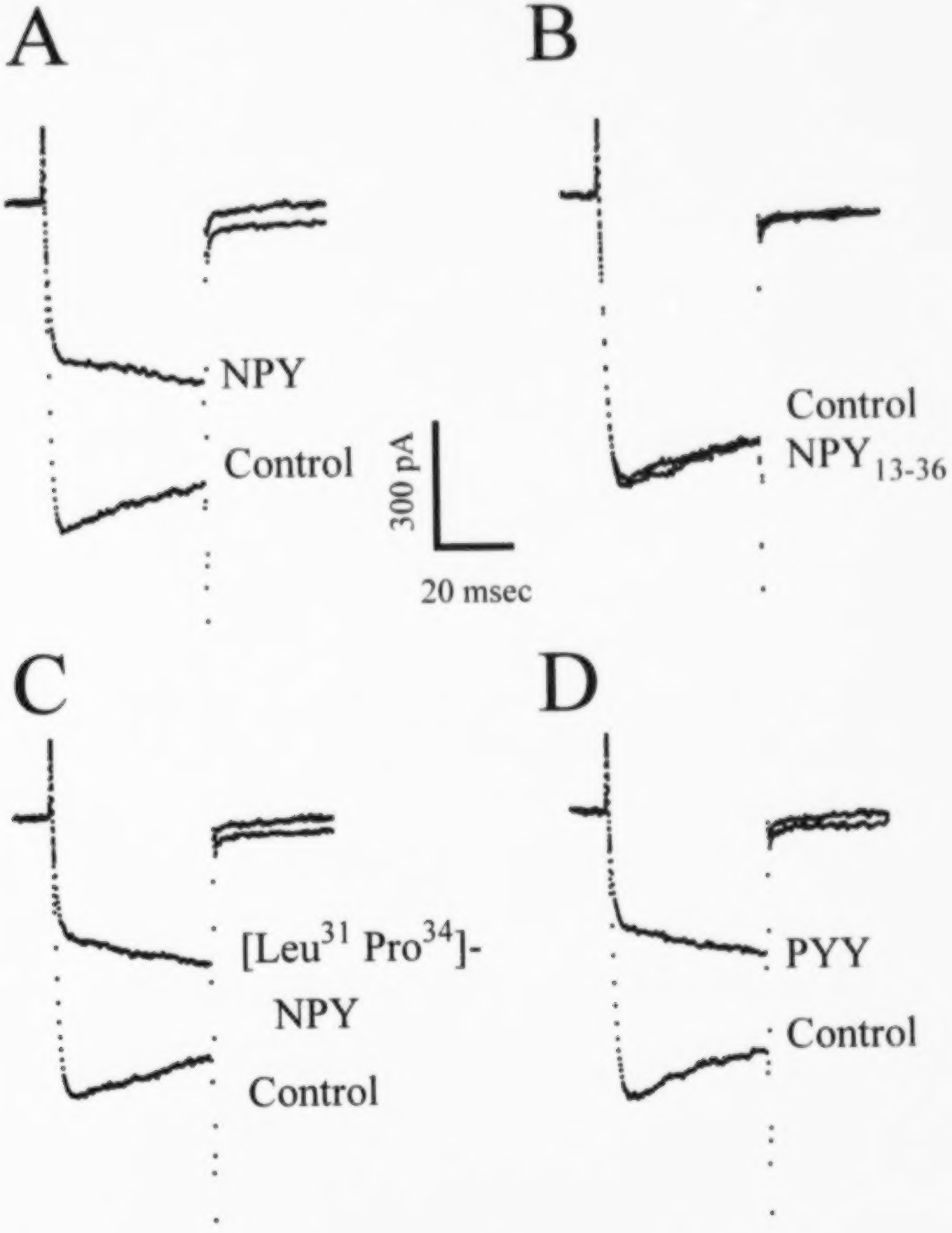
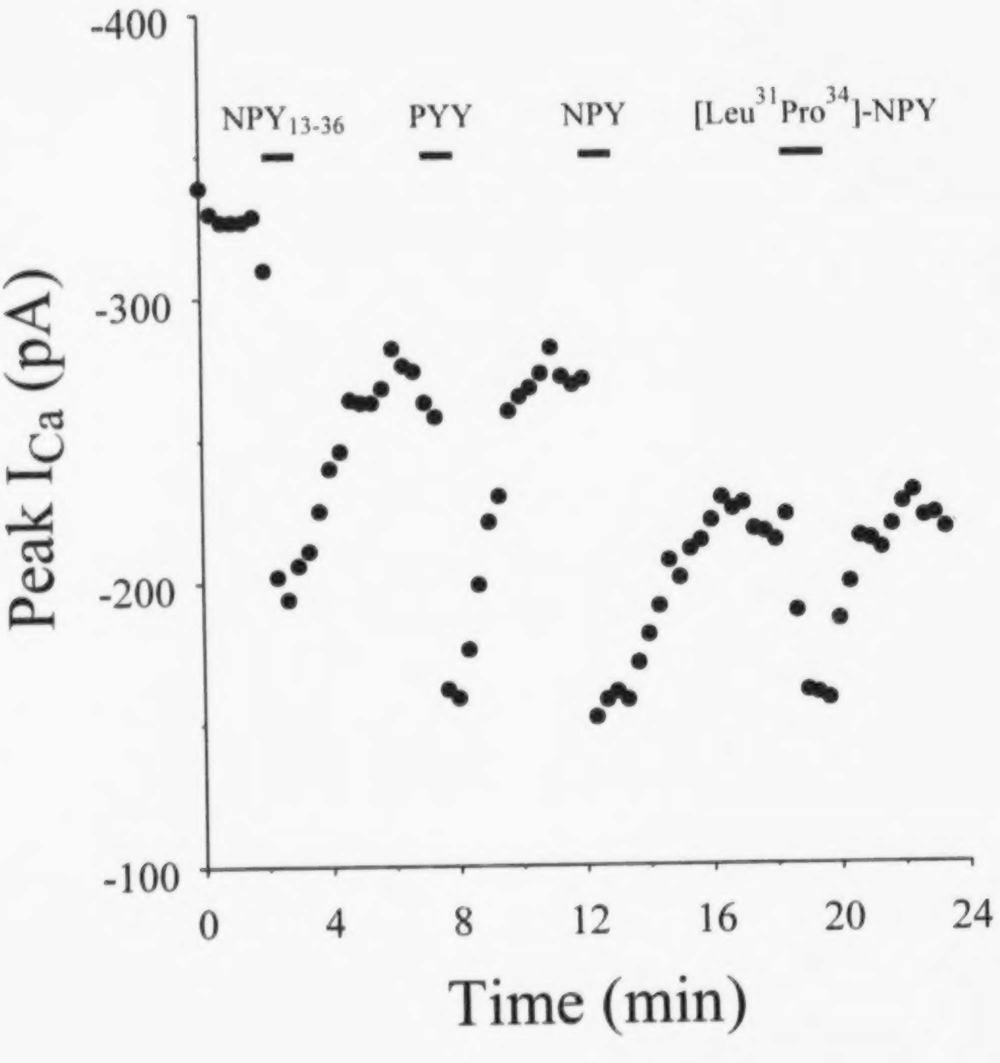

A
B
NPY
Control
300 pA
20 msec
Control
NPY$_{13-36}$
C
D
[Leu^{31} Pro^{34}]-NPY
Control
PYY
Control

Figure 4-4. An Acutely Dissociated DGC is Inhibited by More than One NPY Receptor

The time plot of I_{Ca} is as in Figure 4-3. I_{Ca} in this cell was inhibited by all the agonists tested, including the Y_2-selective NPY_{13-36}. All agonists were applied at a concentration of 1 μM.



NPY13-36
PYY
NPY
[Leu31Pro34]-NPY
Peak ICa (pA)
-400
-300
-200
-100
0
4
8
12
16
20
24
Time (min)

than one NPY receptor subtype must be expressed by this DGC, although this protocol did not distinguish whether Y_2 and/or Y_1 receptors were present on this cell.

Agonist studies are summarized in Figure 4-5. I_{Ca} was inhibited by between 21.8 % and 30.2 % by individual agonists; however there was no significant difference between the four different agonists ($P > 0.30$, Kruskal-Wallis test; Fig. 4-5). The effects of NPY_{13-36} were similar in magnitude to the other agonists, although it was effective in only 31 % (5 of 16 cells) of DGCs which responded to NPY. By contrast, $[Leu_{31}Pro_{34}]$-NPY ($n = 19$) and PYY ($n = 13$) inhibited the I_{Ca} in all NPY-responsive DGCs tested.

NPY Does Not Modulate the Voltage-dependence of I_{Ca} Activation.

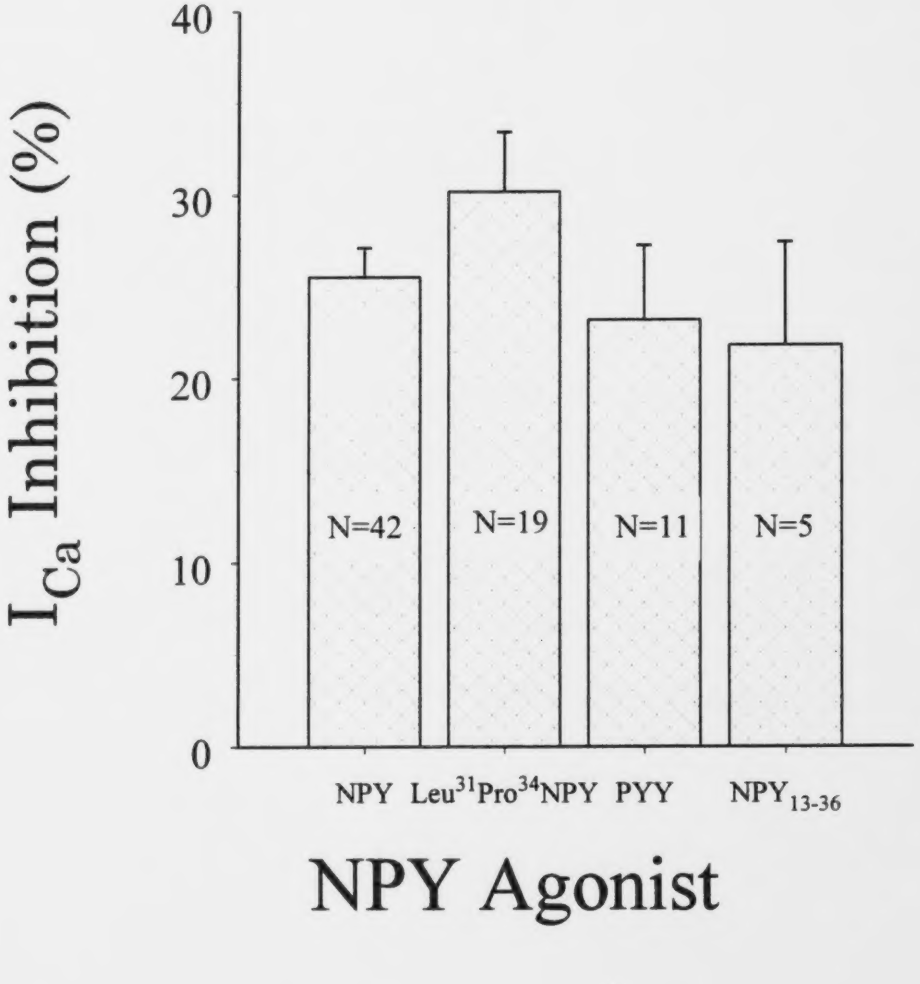
The I_{Ca} of these small cells often ran down rapidly making the construction of a current-voltage (I-V) relationship of the I_{Ca} from a family of voltage steps under several conditions very difficult. To alleviate this problem, we ramped the voltage of the DGC membrane (V_m) from -80 mV to +70 mV over 100 ms (Fig. 4-6). The I_{Ca} of this DGC was inhibited by NPY with no change in the V_m range of I_{Ca} activation or the V_m where I_{Ca} reached a maximum (Fig. 4-6). This DGC was insensitive to NPY_{13-36} (Fig. 4-6). NPY ($n = 7$), $[Leu^{31}Pro^{34}]$-NPY ($n = 4$) and PYY ($n = 3$) inhibited I_{Ca} elicited by voltage ramps in other DGCs without shifting the voltage range of activation or the V_m at which I_{Ca} peaked. We did not see an inhibition of voltage ramped I_{Ca} by NPY_{13-36} ($n = 2$). The I_{Ca} of one voltage ramped DGC did not respond to NPY.

NPY Inhibits an N-type I_{Ca}.

, We next sought to determine the type of I_{Ca} that NPY affected in DGCs. We rarely observed low-voltage activated I_{Ca} in our DGC preparation, therefore we used pharmacological inhibitors of high voltage activated currents in conjunction with NPY

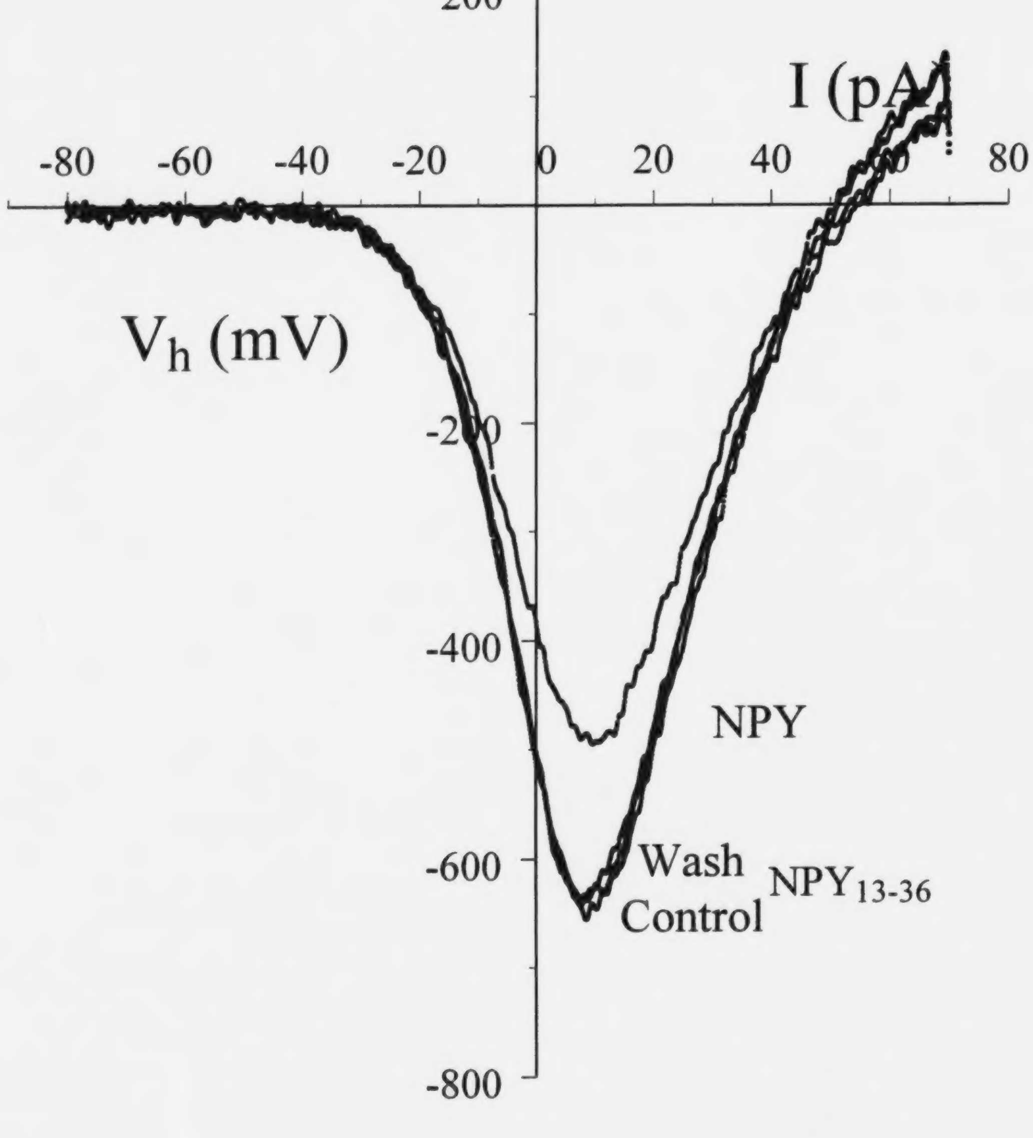
Figure 4-5. Inhibition of DGC I_{Ca} by Receptor-specific Ligands

The histogram of the mean (± SEM) percent reduction of I_{Ca} of all cells responding to the given agonist. The error bars are ± standard error of the mean. The percent reduction of I_{Ca} by the individual agonists are not significantly different ($P > 0.30$, Kruskal-Wallis test). All cells which responded to NPY also responded to [$Leu^{31}Pro^{34}$]-NPY and PYY when tested. Only 31 % of cells responding to NPY also responded to NPY_{13-36} when tested.



I_{Ca} Inhibition (%)
40
30
20
10
0
N=42
N=19
N=11
N=5
NPY
$Leu^{31}Pro^{34}NPY$
PYY
NPY_{13-36}
NPY Agonist

Figure 4-6. NPY Inhibition of I_{Ca} Does Not Shift the Current-Voltage Relationship of I_{Ca}. NPY but not NPY_{13-36} inhibited I_{Ca} elicited by a 100 ms voltage ramp from -80 mV to +70 mV. NPY did not shift the voltage range of I_{Ca} activation or the voltage at which I_{Ca} reached its maximum.



200
I (pA)
-80
-60
-40
-20
0
20
40
80
V$_h$ (mV)
-200
-400
NPY
-600
Wash
Control
NPY$_{13-36}$
-800

in order to determine the I_{Ca} in DGCs that NPY inhibits., We either applied NPY or [Leu^{31}Pro^{34}]-NPY in the absence or presence of the L-type I_{Ca} inhibitor nimodipine (1 to 5 μM) or the N-type I_{Ca} inhibitor ω-conotoxin GVIA (ω-CTX GVIA, 3 to 5 μM). The peak DGC calcium current was inhibited by $44 \pm 6\%$ (n = 9) by ω-CTX GVIA and by $23 \pm 6\%$ (n = 7) by nimodipine. The time plot of Figure 4-7 demonstrates that ω-CTX GVIA completely occluded the [Leu^{31}Pro^{34}]-NPY inhibition of I_{Ca} in this DGC. The same observation was made in 7 other cells inhibited by NPY and 1 other cell inhibited by [Leu^{31}Pro^{34}]-NPY. The data are summarized in Figure 4-8. Figure 4-8A illustrates that ω-CTX-GVIA completely occludes the inhibition of I_{Ca} by NPY (n = 7). In contrast, nimodipine did not significantly affect the inhibition of I_{Ca} by NPY (Fig. 4-8A, n = 7).

However, the magnitude of I_{Ca} inhibition by NPY is reduced $32 \pm 10\%$ (N = 8) in the second application of NPY to the same neuron (Fig. 4-8B). Therefore, comparing the results of the application of NPY in the presence of nimodipine will underestimate the inhibition of I_{Ca} by NPY in nimodipine subsequent to the first application (Fig. 4-8A, right). We can correct the value of I_{Ca} inhibition in the presence of nimodipine for the intrinsic 32% reduction in the response to NPY we observed in the absence of any Ca^{2+} channel blockers. In addition, if NPY does not affect the nimodipine-sensitive I_{Ca}, then it should inhibit a larger percentage of the current remaining after the application of nimodipine than in control. This can be calculated for each cell without further assumptions from the amount of I_{Ca} blocked by nimodipine and the initial response to the NPY agonist. This predicted inhibition of total I_{Ca} by NPY in the presence of nimodipine is shown on the left of Figure 4-8C, while the observed inhibition of I_{Ca} by nimodipine, corrected for the rundown of the NPY response is shown on the right. There was no

Figure 4-7. NPY Inhibits an N-type I_{Ca} in Acutely Dissociated DGCs.

Details of the experiment are as Fig. 4-3. The Y_1 agonist [$Leu^{31}Pro^{34}$]-NPY inhibition I_{Ca}. This response was occluded by application of 5 μM ω-conotoxin GVIA. Similar findings were seen in 7 cells for NPY and 2 other cells for [$Leu^{31}Pro^{34}$]-NPY.

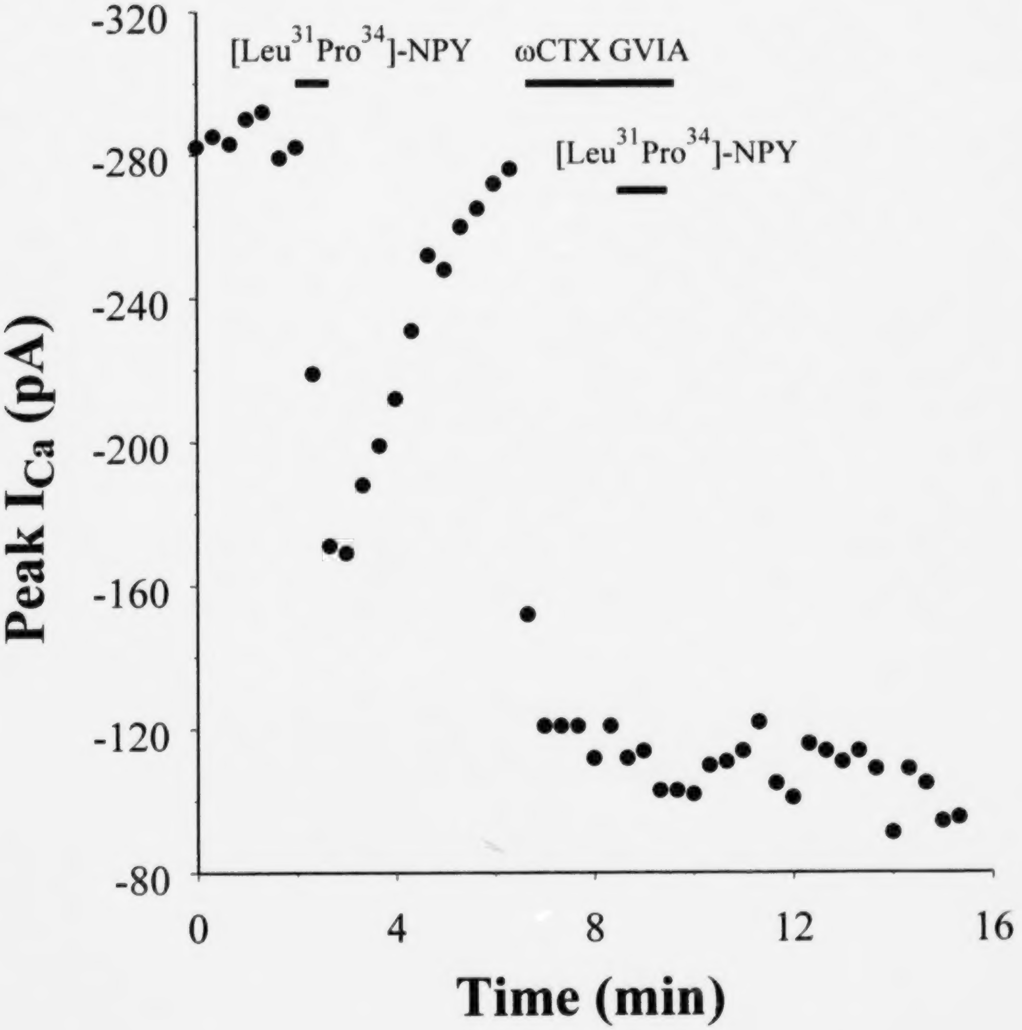
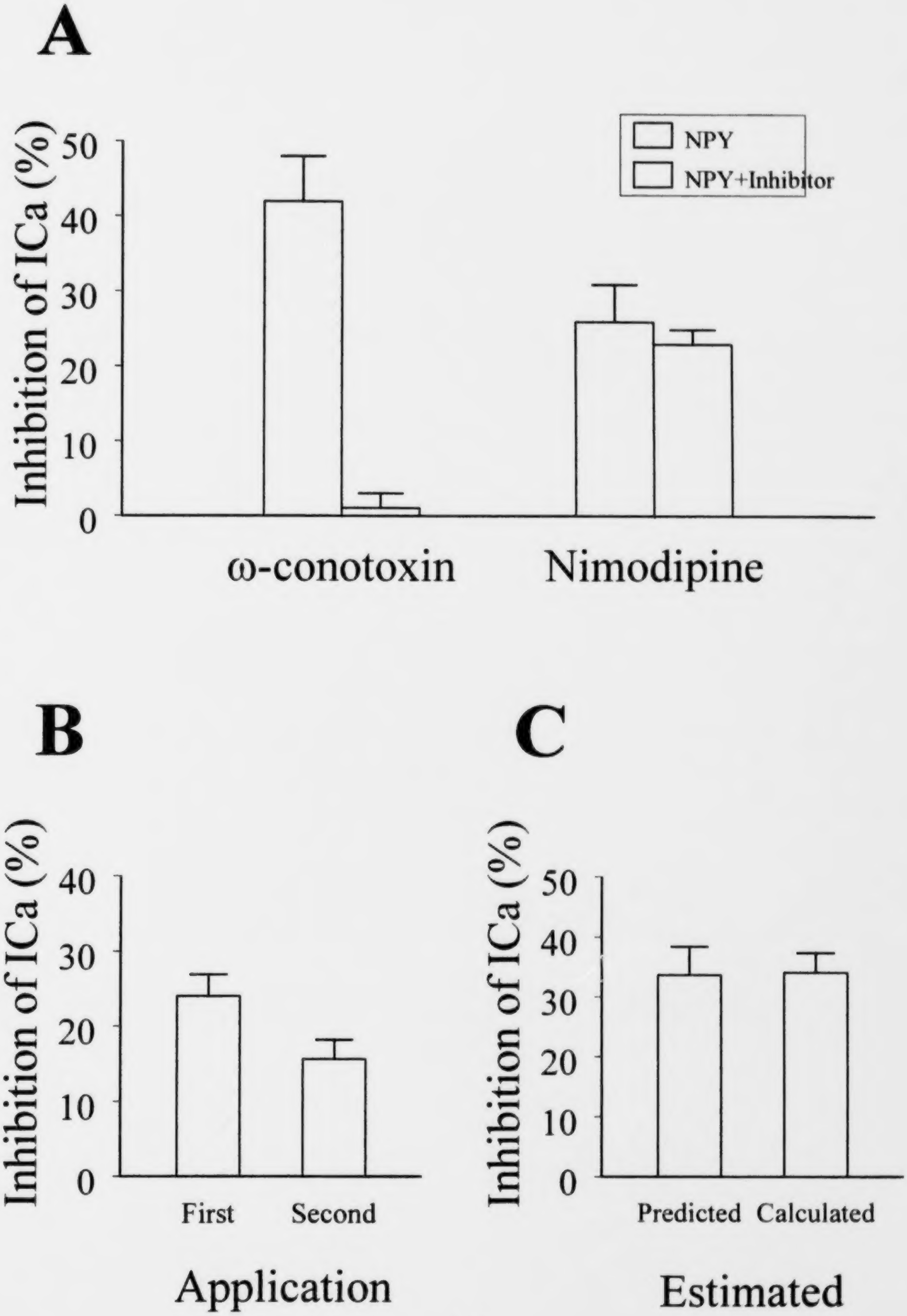

[Leu^{31}Pro^{34}]-NPY
ωCTX GVIA
[Leu^{31}Pro^{34}]-NPY
Peak I_{Ca} (pA)
-320
-280
-240
-200
-160
-120
-80
0
4
8
12
16
Time (min)

Figure 4-8. NPY only inhibits N-type I_{Ca} in Acutely Dissociated DGCs.

A. A bar graph showing that inhibition of I_{Ca} by NPY in 7 neurons (42 ± 6%, cross-hatched bar, left) is completely occluded by 3 μM ω-CTX-GVIA (1 ± 2%, open bar left, $P < 0.02$ Wilcoxon signed-ranks test). Inhibition of I_{Ca} by NPY (26 ± 5%, cross-hatched bar right) is not affected by treatment with 3 μM nimodipine (23 ± 6%, open bar right, $N = 7$, $P > 0.59$ Wilcoxon signed-ranks test). B. The inhibition of I_{Ca} produced in 8 different cells by a second application of NPY (16 ± 3%, cross-hatched bar right) is reduced by 32 ± 10% when compared to the inhibition produced by the first application of NPY (24 ± 3%, open bar left, $N = 8$, $P < 0.02$, Wilcoxon signed-ranks test). C. Estimated inhibition of I_{Ca} by NPY. If NPY were to inhibit only the nimodipine-insensitive portion of I_{Ca}, then it should have a proportionally greater effect on I_{Ca} remaining after nimodipine, in this case 34 ± 5% (predicted, hatched bar), based on the observed proportion of nimodipine-sensitive I_{Ca} in these cells. The NPY response in nimodipine, when corrected for the rundown of the response to the second application is calculated to be 34 ± 3 % (Corrected, open bar). These two values are not statistically significant ($P > 0.39$, Wilcoxon signed-ranks test).

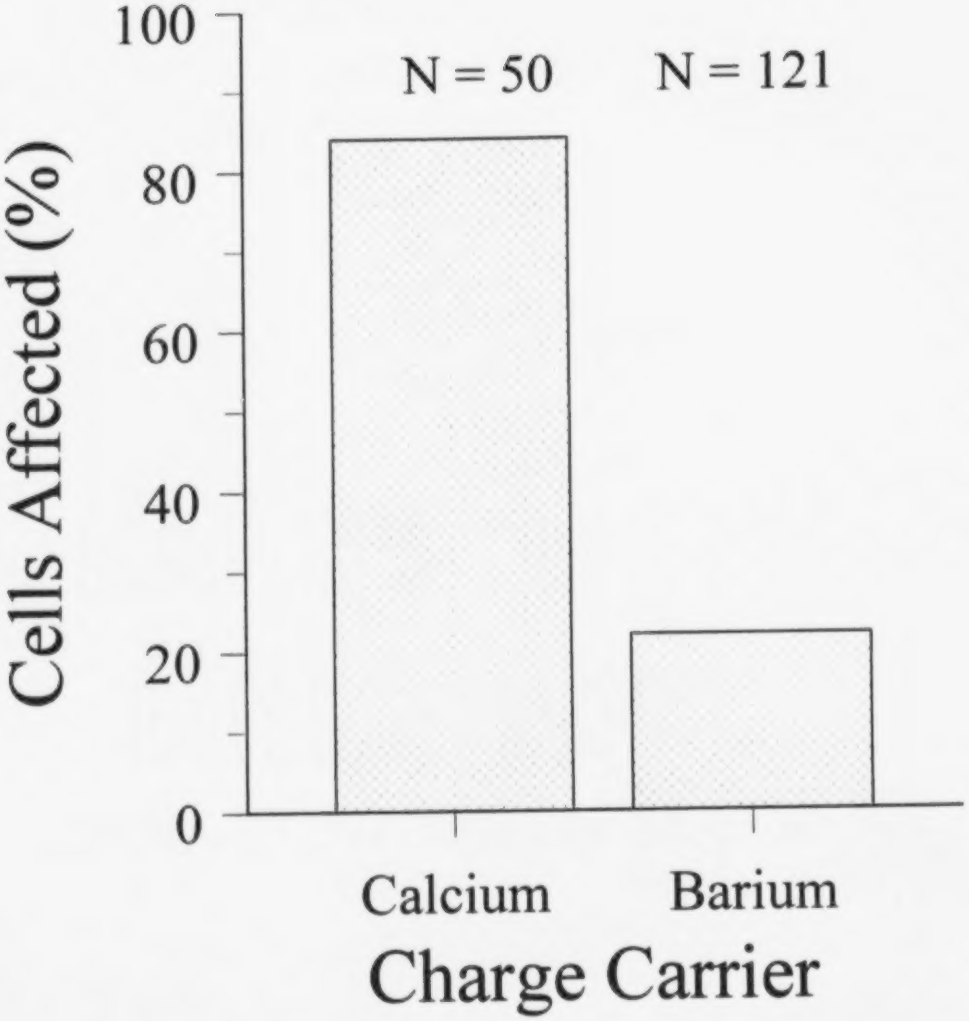

A
Inhibition of ICa (%)
50
40
30
20
10
0
NPY
NPY+Inhibitor
ω-conotoxin
Nimodipine
B
Inhibition of ICa (%)
40
30
20
10
0
First
Second
Application
C
Inhibition of ICa (%)
50
40
30
20
10
0
Predicted
Calculated
Estimated

significant difference between the two (Wilcoxon signed-ranks test, $P > 0.73$), suggesting that there is indeed no effect of nimodipine on the I_{Ca} reduced by NPY receptors in these neurons.

Inhibition of I_{Ca} by NPY is Ca^{2+}-sensitive.

In experiments on dissociated cells recorded with conditions that favored a moderate resting $[Ca^{2+}]_i$ (Ca^{2+} as the charge carrier and minimal Ca^{2+} buffering in the pipette solution), 42 of 50 DGCs responded to NPY (Fig. 4-9). By contrast, under experimental conditions favoring low $[Ca^{2+}]_i$ (Ba^{2+} as the charge carrier and strong Ca^{2+} buffering in the pipette), NPY inhibited the I_{Ca} of only 23 % of DGCs ($N = 121$) (Fig. 4-9). In contrast, application of 10 μM baclofen inhibited the barium current in 81 % of such DGCs ($N = 27$; data not shown), indicating that another agonist was capable of inhibiting I_{Ca} in these cells under these circumstances.

Figure 4-9. NPY Inhibition of I_{Ca} in Acutely Dissociated DGCs is Sensitive to Calcium. The bar on the left (Ca^{2+}) indicates the percentage of NPY-responsive cells recorded with 5 mM Ca^{2+} in the extracellular solution as the charge carrier and low (0.1 mM BAPTA) intracellular Ca^{2+} buffering. The bar of the right (Ba^{2+}) indicates the percentage of NPY-responsive cells recorded with 5 mM Ba^{2+} in the extracellular solution as the charge carrier and high (20 mM BAPTA with no added Ca^{2+}) intracellular Ca^{2+} buffering. A much larger proportion of cells responded to NPY when Ca^{2+} was the charge carrier and the $[Ca^{2+}]_i$ was weakly buffered.


100
80
60
40
20
0
Cells Affected (%)
N = 50
N = 121
Calcium
Barium
Charge Carrier

Discussion

The main findings of this study are that NPY inhibits an N-type calcium channel in DGCs of the rat hippocampus, predominantly by activating a Y_1 receptor, and that this inhibition results in significantly smaller changes to $[Ca^{2+}]_i$ during electrical activity. To our knowledge this is the first report of NPY inhibiting a I_{Ca} of a central neuron, and the first report of NPY inhibiting a I_{Ca} by the activation of a Y_1 receptor. Furthermore, the Y_1 receptor inhibition of I_{Ca} observed here in DGCs is in sharp contrast to the usual potentiating or activating functions which have been associated with the activation of Y_1 receptors in previous studies of other cell types (Michel, 1991; Wiley *et al.*, 1993; Xiong *et al.*, 1993).

We had originally hypothesized that NPY would modulate the $[Ca^{2+}]_i$ or Ca^{2+} influx in rat hippocampal DGCs, based on previous findings that Y_1 receptors were often coupled to release of $[Ca^{2+}]_i$ or potentiated Ca^{2+} influx in other cell types (Michel, 1991; Wahlestedt and Reis, 1993; Wiley et al., 1993). The imaging experiments showed no change in resting $[Ca^{2+}]_i$ of DGCs following application to NPY, and an inhibition of depolarization-induced rises in $[Ca^{2+}]_i$. We did not observe a change in the holding current during the application of NPY to intact cells in slices, making it most unlikely that NPY activates or potentiates a K^+ channel, (Zidichouski *et al.*, 1990), in agreement with previous observations (Klapstein and Colmers, 1993). Further support for this conclusion is provided by previous observations that NPY did not affect the afterhyperpolarization following APs of DGC's in hippocampal slices (Klapstein and Colmers, 1993). The simplest explanation for these observations is that NPY inhibits the VDCCs in DGCs, and the experiments on the acutely dissociated DGCs support this conclusion.

In the NPY-responsive DGCs to which selective agonists were applied, [Leu^{31}Pro^{34}]NPY and PYY always inhibited I_{Ca}. In contrast, NPY$_{13-36}$ was inactive in the majority of NPY-sensitive DGCs. Since NPY$_{13-36}$ is inactive at Y_1 receptors, with the highest activity at Y_2 and some activity at Y_3 receptors (Michel *et al.*, 1991; Foucart *et al.*, 1993) it can be concluded that, in the majority of DGC's, I_{Ca} is inhibited via a Y_1 receptor-mediated mechanism. In DGCs which responded to NPY$_{13-36}$, NPY must be activating more than one receptor, but no single agonist used here is selective for any one receptor subtype (Michel, 1991). The exact combination of receptors present on these DGCs may involve the combination of any two or all three receptor subtypes. Since all NPY-sensitive DGCs appear to be responsive to [Leu^{31}Pro^{34}]-NPY, we believe that Y_1 receptors are likely to be present on the cells that are responsive to NPY$_{13-36}$. These observations agree with binding studies showing a preponderance of Y_1 binding sites in rat dentate gyrus, with far fewer Y_2 sites (Dumont *et al.*, 1990, 1993; Aicher *et al.*, 1991).

Our data demonstrate that NPY inhibits N-type I_{Ca} of rat DGCs. In every case, the NPY- or [Leu^{31}Pro^{34}]NPY-mediated inhibition of I_{Ca} was entirely occluded by ω-CTX GVIA but not by nimodipine. The amount of I_{Ca} inhibition by NPY, in the presence of nimodipine (corrected for the rundown of the response), matches the predicted magnitude of inhibition of I_{Ca} if NPY were to have no effect on L-type VDCCs. NPY inhibits an N-type current in numerous other cell types (Bleakman *et al.*, 1993; Colmers and Bleakman, 1994). However, in some cells of the PNS, I_{Ca} inhibition is mediated by a Y_2 receptor (Bleakman *et al.*, 1991; Toth *et al.*, 1993; Wiley *et al.*, 1993). Y_1 receptor activation, by contrast, usually results in a potentiating effect on I_{Ca}. For example, in rat nodose ganglia, Y_1 receptor activation potentiates the same I_{Ca} which Y_2 receptor activation inhibits (Wiley *et al.*, 1993). Y_1 receptor activation potentiates α_1 adrenoceptor actions in vascular smooth muscle (Zukowska and Wahlestedt, 1993), and potentiates L-type VDCCs in vascular smooth muscle (Xiong *et al.*, 1993). Such Y_1-mediated potentiations are in contrast to the observed Y_1 receptor action in the rat DGC. It is interesting to note that ω-CTX GVIA inhibited only 20% of the I_{Ca} in DGCs isolated from the guinea pig hippocampus (Eliot and Johnston, 1994) compared to the 44% inhibition reported here in the rat, suggesting a substantial species difference in these cells. In the majority of DGCs, NPY inhibited I_{Ca} without changing the timecourse of I_{Ca}. However, in about 25% of DGCs, NPY inhibition of I_{Ca} was accompanied by a slowing of the timecourse of activation, as has been seen elsewhere (Bean, 1989; Hille, 1994). Here, NPY did not change the voltage range at which I_{Ca} activated or the V_m at which I_{Ca} reached a maximum. Previous investigations in other types of neurons have demonstrated that other neuromodulators can inhibit I_{Ca} by two distinct mechanisms as NPY appears to do in DGCs (Beech *et al.*, 1992; Formenti *et al.*, 1993; Luebke and Dunlap, 1994; Toselli and Taglietti, 1993). Most of the DGC's here respond to NPY without an apparent alteration in the timecourse of their I_{Ca}, as others have observed in a proportion of chick sensory neurons (Luebke and Dunlap, 1994). Because of the relative rarity of the altered timecourse, we did not perform the depolarizing prepulse experiments necessary to distinguish between the two mechanisms (e.g., Luebke and Dunlap, 1994), and further studies are required to elucidate the pathways by which the receptor couples to the calcium channels.

Inhibition of I_{Ca} in rat DGCs by NPY appears also to be sensitive to $[Ca^{2+}]_i$. Recording conditions favoring low $[Ca^{2+}]_i$ (i.e. high intracellular BAPTA concentrations) greatly reduces the proportion of cells responding to NPY. Only 23% of DGCs in which Ba^{2+} was the charge carrier and internal Ca^{2+} was strongly buffered responded to NPY. However, baclofen inhibited I_{Ba} in 81 % of DGCs recorded under the same conditions. When recording conditions were changed to favor higher $[Ca^{2+}]_i$, (i.e. low intracellular BAPTA concentrations) the proportion of DGCs responding to NPY increased to 84 %. Recent studies have shown that inhibition of I_{Ca} in neurons is caused by a number of distinct mechanisms (reviewed in Hille, 1994). One such mechanism is sensitive to $[Ca^{2+}]_i$. Buffering $[Ca^{2+}]_i$ to very low concentrations either blocks or reduces the inhibition of I_{Ca} by metabotropic glutamate receptors in cultured hippocampal neurons (Lester and Jahr, 1990) and by M_1 ACh and angiotensin II receptors in rat SCG neurons (Beech *et al.*, 1991, Shapiro *et al.*, 1994). The mechanism of this Ca^{2+}-or BAPTA- sensitivity is unknown; however, in rat SCG neurons the Ca^{2+}-sensitivity does not involve releasable pools of Ca^{2+} (Beech *et al.*, 1991). More recently, it has been shown in chick sensory neurons that there are two separate pathways by which N-type I_{Ca} can be inhibited by activation of the α_2 adrenoceptor, one of which does not alter the timecourse of current activation, and which involves protein kinase C (Diversé-Pierliussi *et al.*, 1995). It is possible that the NPY inhibition of I_{Ca} in rat DGCs may employ similar coupling mechanisms.

Although NPY inhibits an N-type VDCC in rat DGCs via the activation of Y_1 receptors, the biological purpose of this action is not certain. Regulation of Ca^{2+} influx in neurons can alter the excitability of the neuron (Madison and Nicoll, 1984). NPY has no evident effect on the excitability of DGCs (Klapstein and Colmers, 1993). However, recent studies have shown that Ca^{2+} influx through L-type VDCCs can induce the expression of specific immediate early genes and thus presumably regulate neuronal gene expression (Morgan and Curran, 1986; Sheng *et al.*, 1990; Murphy *et al.*, 1991; Ghosh *et al.*, 1994). DGCs are remarkably plastic in the expression of their phenotype, altering the transmitters they express depending on previous excitation (Morris *et al.*, 1988; Goodman and Sloviter, 1993). It may be that NPY regulates activity-dependent gene expression in DGCs. Alternatively or in addition, NPY could act to prevent damaging levels of Ca^{2+} influx under conditions of elevated levels of excitation.

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Chapter 5

General Discussion

This chapter will briefly review the three preceding chapters and then attempts to describe a function for NPY in the hippocampus.

Chapter 2 examined the possibility that NPY may act to potentiate NMDA receptor activity in area CA3 of the rat dorsal hippocampus. NPY did not modulate the current evoked by NMDA in CA3 pyramidal cells of rat hippocampal slices. NPY was shown to be active in the slice at the site of the cell being recorded, as NPY potently inhibited the excitatory postsynaptic current evoked by the stimulation of the mossy fiber input from the dentate gyrus. Other investigators have shown NPY and some agonists appear to potentiate responses to NMDA (Monnet *et al.*, 1990, 1991, 1992a,b; Roman *et al.*, 1991). These investigators demonstrated that the effects of NPY were due to the activation of a receptor sensitive to sigma receptor antagonists (Monnet *et al.*, 1990, 1992a,b; Roman *et al.*, 1991). However, the NPY agonist profiles differ between these studies and to those identified with *in vivo* binding (Bouchard *et al.*, 1993). For example, in a study of NPY's ability to potentiate NMDA-induced activity in CA3 pyramidal neurons of the dorsal hippocampus of the rat, Monnet and colleagues (1992a) found NPY_{13-36} to be active on NMDA responses. This is in contrast to the inability of NPY_{13-36} to bind to sigma receptors *in vivo* in the mouse hippocampus (Bouchard *et al.*, 1993). Furthermore, NPY_{2-36} has no effect on NMDA-induced activity in CA3 pyramidal cells but has been shown to bind to sigma receptors *in vivo* (Bouchard *et al.*, 1993). Inconsistencies also appear in comparing the effect of NPY agonists on two different assays for NMDA activity. This is seen for the effect of PYY, where PYY potentiates the NMDA-induced release of NE from rat hippocampal slices (Roman *et al.*, 1991) However, PYY does not potentiate NMDA-induced activity of CA3 pyramidal cells but instead acts as an antagonist to NPY agonists which potentiate these NMDA-induced responses (Monnet *et al.*, 1992a). Therefore, as mentioned in the introduction, the evidence for NPY acting at sigma receptors and potentiating the activity of NMDA receptors is riddled with controversy and inconsistencies.

Because of the observation that NPY agonists can displace sigma ligand binding *in vivo* (Bouchard *et al.*, 1993) but not *in vitro* (Quirion *et al.*, 1991), Quirion and colleagues have suggested that the *in vivo* displacement of sigma ligands by NPY and its agonists is not due to direct binding to the sigma receptor. They attribute this result to NPY somehow causing the release of an endogenous sigma ligand or NPY activating a second messenger system which indirectly alters sigma receptor binding. A similar hypothesis could explain our results in comparison to those of Monnet *et al*. (1990, 1991, 1992a,b). The results of chapter 2 clearly indicate that NPY does not directly modulate the NMDA receptor. The contradictory results of the other investigators could be explained by an effect of NPY modulating the activity of the hippocampal circuitry *in vivo* to cause the release of some other modulator which in turn would modulate the NMDA receptor via the activation of the sigma receptor. However, the data presented in chapter 2 does not directly address the inconsistencies in agonist profiles associated with the putative NPY/sigma receptors or whether NPY can directly activate a sigma receptor. The data in Chapter 2 is consistent with NPY not directly acting at a sigma receptor which is in agreement with other investigations (Tam and Mitchell, 1991; Quirion *et al.*, 1991). This controversy awaits the synthesis of selective NPY receptor antagonists which will permit an examination of whether the putative effects of NPY on sigma receptors are indirectly due to the activation of Y_1, Y_2, or Y_3 receptors, as yet uncharacterized NPY receptor or perhaps due to a non-receptor mediated action of NPY, as has been hypothesized elsewhere (Grundemar *et al.*, 1993). Certainly, the burden of proof remains on the proponents of NPY acting as an agonist on sigma receptors, as they have been unable to demonstrate in their experiment even the most potent and reproducible effect of NPY in the hippocampus, namely, presynaptic inhibition.

Chapter 3 deals again with excitatory synaptic transmission onto CA3 pyramidal neurons of the rat hippocampal slice. Specifically, the site and mechanism of NPY action was investigated. The main finding was that NPY presynaptically inhibited the release of glutamate onto CA3 pyramidal cells, since NPY did not inhibit the amplitude or interval distributions of mEPSCs despite its inhibitory action on the interval distribution of sEPSCs. This is in agreement with glutamate release studies from slices of rat hippocampus which show that NPY inhibits the release of elevated extracellular K^+ induced-release of glutamate (Greber *et al.*, 1994). The effect of NPY was abolished with the elimination of presynaptic APs by treating the slice with TTX. This suggests that NPY is not acting by directly inhibiting the transmitter release machinery, but at an impulse-dependent synaptic release process before this point. In contrast to these findings, presynaptic inhibition studies at the neuromuscular junction have shown adenosine to inhibit the release of acetylcholine by directly inhibiting the protein machinery responsible for mediating exocytosis of transmitter containing vesicles from the presynaptic terminal (Silinsky, 1984). Furthermore, both $GABA_B$ and adenosine receptor activation inhibit the frequency of mEPSCs but not their amplitude distributions in CA3 pyramidal neurons in slice cultures of rat hippocampus which suggests again that these neurotransmitters inhibit the release of glutamate in part by directly inhibiting the proteins mediating transmitter exocytosis (Scanziani *et al.*, 1992). However, like the actions of NPY on mEPSCs of rat hippocampal slices, α_1 receptor activation in hippocampal slice cultures produces no effect on the amplitudes or frequency of mEPSCs in CA3 pyramidal cells despite its ability to inhibit excitatory synaptic transmission (Scanziani *et al.*, 1993). Thus it appears that neuromodulators have multiple presynaptic mechanisms of action to presynaptically inhibit the release of neurotransmitter.

The requirement of presynaptic impulses for NPY to inhibit transmitter release suggests a number of possible mechanisms of action. First, NPY could be inhibiting the excitability of the presynaptic terminal or presynaptic axons by the activation of a K^+ current, inhibition of the Na^+ current or a shunting current, reducing the probability of AP invasion into the terminal. Second, NPY may increase or potentiate a K^+ current responsible for the repolarization of the AP and thus limit the amount of Ca^{2+} entry into the terminal. Third, one or more Ca^{2+} currents responsible for transmitter release may be directly inhibited. Four, kinetically fast Ca^{2+} buffering in the terminal may be increased or the receptor affinity for the Ca^{2+} trigger could be decreased. The results of chapter 3 cannnot distinguish between any of these possible mechanisms. However, if data from studies of NPY at other synapses are generalized, the number of possible mechanisms can be reduced in favour of others. In the CA1 region of the rat hippocampus, NPY also appears to inhibit the release of glutamate onto CA1 pyramidal neurons (Colmers *et al.*, 1985, 1987, 1988). In this area, NPY does not appear to affect the excitability of the presynaptic axon or terminal (Colmers *et al.*, 1988). This was determined by measuring the presynaptic volley extracellularly, the activity of the cell bodies of the presynaptic neurons and the activity of the presynaptic cells by antidromic activation (Colmers *et al.*, 1988). In region CA1, NPY does not appear to act through the modulation of a 4-AP-sensitive K^+ channel (Colmers *et al.*, 1988). Therefore, NPY in area CA1 was hypothesised to most likely act by inhibiting presynaptic Ca^{2+} influx into the presynaptic terminal (Colmers *et al.*, 1985; 1987; 1988; Klapstein and Colmers, 1992). NPY has been definitively shown to inhibit the release of neurotransmitter from sympathetic neurons in coculture with atrial myocytes of the rat by the inhibition N-type VDCCs in the presynaptic terminal (Toth *et al.*, 1993). Therefore, if NPY has a common mechanism of action for presynaptic inhibition throughout the CNS and PNS of mammals, the results presented in Chapter 3 are consistent with NPY presynaptically inhibiting the release of glutamate onto CA3 pyramidal neurons by inhibiting presynaptic Ca^{2+} channels.

CA3 pyramidal cells receive excitatory synaptic input from a number of different sources which synapse onto different regions of the dendritic tree. Klapstein and Colmers (1993) have demonstrated that the mossy fiber input, the associational input and the commissural inputs to CA3 pyramidal neurons of the rat hippocampal slice are all inhibited by NPY. The actions of NPY onto the perforant path input to CA3 has not been directly investigated. The investigations in chapter 3 could not discriminate between these separate inputs and were analysed as a whole. However, since the entire distribution of mEPSC amplitudes was unaffected by NPY, it would appear that NPY acts through a similar mechanism at all the separate inputs to CA3.

Chapter 4 describes the effect of NPY in the dentate gyrus of the rat. It was shown that NPY via the activation of a Y_1 receptor inhibits the influx of Ca^{2+} through N-type VDCCs in the soma and dendrites of rat DGCs. The physiological function of this inhibition has not been elucidated. One possible function could be to control the excitability of the DGC. Injection of the Ca^{2+} chelators EGTA or BAPTA into DGCs reduces the spike frequency adaptation of the DGC in response to a depolarizing current injection (Blaxter *et al.*, 1989; Niesen *et al.*, 1991; Staley *et al.*, 1992). In contrast, the AP duration and the single spike AHP was not affected by Ca^{2+} chelators (Blaxter *et al.*, 1989; Niesen *et al.*, 1991; Staley *et al.*, 1992). This suggests that Ca^{2+} influx may control the excitability of the neuron to prolonged depolarizations but not the repolarization of the action potential. Thus NPY may modulate the excitability of the DGC by inhibiting Ca^{2+} influx and thereby reducing accommodation. NE has been shown to inhibit accommodation in DGCs (Haas and Rose, 1987), however, NE actually potentiates Ca^{2+} influx in these cells (Gray and Johnston, 1987), suggesting that NE acts by directly inhibiting the current responsible for the accommodation. Preliminary evidence suggests that NPY does not affect accommodation in rat DGCs (Klapstein and Colmers, 1993), but further investigation into NPY and accommodation in DGCs is required.

An alternative hypothesis involves intracellular Ca^{2+} regulation of gene expression (Ghosh *et al.*, 1994). Many investigators have proposed that influx of $[Ca^{2+}]_i$ may transduce neuronal activity through the activation of immediate-early genes into longer term changes in activity-dependent gene expression of neuronal products such as neurotransmitter receptors and enzymes responsible for the synthesis of neurotransmitter (Ghosh *et al.*, 1994). In primary cultures of rat hippocampal neurons, it has been shown that Ca^{2+} influx through different types of Ca^{2+} channels results in the activation of different subsets of promoter response elements of immediate-early genes (Bading *et al.*, 1993; Murphy *et al.*, 1991). This difference was not due to differences in the absolute levels of Ca^{2+} entering the cell through the separate Ca^{2+} channels, but may be the result of the distinct locations of the individual Ca^{2+} channels in the cell membrane (Ghosh *et al.*, 1994). Dentate granule cells of the rat have been shown to be very plastic in terms of their phenotypical expression of neurotransmitters in an activity-dependent manner (Goodman and Sloviter, 1993; Morris *et al.*, 1988). Therefore, NPY release may modulate activity-dependent gene expression in DGCs by modulating Ca^{2+} influx through N-type channels, a distinct subset of Ca^{2+} channels in the rat DGC.

What does NPY do in terms of function in the hippocampus? This question is difficult to answer because the function of the hippocampus is still not known for certain (Eichenbaum et al., 1992) and the conditions by which NPY is released in the hippocampus are still unknown.

As mentioned in the introduction, the hippocampus displays several behaviourally-dependent rhythms (Lopez da Silva *et al.*, 1990). Theta rhythm (4-12 Hz) is seen during attentive states such as exploratory behaviour while gamma rhythm is seen interspersed with theta and is thought to be involved in temporally binding together spatially segmented but perceptually linked neurons to form the percept (Gray *et al.*, 1989). Theta rhythm appears to be the result of synchronizing inhibitory interneurons of the hippocampus via the rhythmic input of synchronous septal GABAergic input (Freund and Antal, 1988; Ylinen *et al.*, 1995). NPY in the hippocampus should not affect this rhythm as it has no effect on inhibitory synaptic transmission in the hippocampus (Klapstein and Colmers, 1993). Furthermore, the gamma rhythm appears to rely on the circuitry of the dentate gyrus, particularly the perforant path input and the inhibitory neurons of the hilus (Buzsaki *et al.*, 1987; Bragin *et al.*, 1995). Although an effect for NPY on hilar neurons has not been investigated, the other requirements for gamma (ie. perforant path input and inhibitory synaptic transmission) are unaffected by NPY (Klapstein and Colmers, 1993). Therefore, NPY would not appear to be able to alter behaviourally relevant rhythms of the hippocampus. However, the excitatory synaptic transmission of information through the hippocampus could be potently suppressed by NPY. Potent presynaptic inhibition onto CA3 pyramidal neurons and CA1 pyramidal neurons would suppress the transfer of information through both regions only permitting very high frequency information to be passed through. The exact pathways which are inhibited by NPY are not completely known. Although the perforant path onto the DGCs is unaffected, the input from the perforant path onto CA3, CA1 and subiculum is unknown. If these pathways are also unaffected by NPY, then information may be passed through this more direct perforant pathway.

As mentioned, the conditions for the release of NPY is unknown. This is very important to understanding the function of NPY in the hippocampus. If high frequency activity of the NPY containing interneurons is required for release of NPY, like in the sympathetic nervous system (Wahlestedt and Reis, 1993), then NPY may not play an important role during normal functioning of the hippocampus. NPY may only play a modulatory role under situations of very high activity in the hippocampus in which NPY would potently suppress excitatory synaptic transmission without affecting inhibition. In this way NPY may act to prevent pathological hyperexcitability leading to epileptic seizure. In the dentate gyrus, NPY would modulate the gene expression during such periods to possibly prevent further excitation on a more long term basis. The suppression of Ca^{2+} influx may also prevent cytotoxic damage caused by this influx of Ca^{2+}. Thus, NPY may play a protective role in the hippocampus during periods of hyperexcitability. Finally, other possible cellular actions of NPY have still to be investigated. As previously mentioned, the perforant inputs to CA3, CA1 and the subiculum have still to be investigated. This may be difficult in the slice preparation as inputs to these regions are not laminar and are for the most part likely to be severed in the slicing procedure. The inner-one third of the molecular layer of the dentate gyrus contains most of the NPY binding in the dentate gyrus. This area also contains the inputs from hilar cells to the DGCs and the dendrites of some of the hilar neurons. Thus an action of NPY on synaptic transmission of these inputs onto DGCs and hilar neurons deserves more attention. CA3 pyramidal neurons have recently been shown to have collaterals back to the dentate gyrus (Ishizuka *et al.*, 1990; Li *et al.*, 1994; Scharfman and Schwartzkroin, 1988; Scharfman, 1991; 1995). Perhaps this input like the commissural input, the associational input (Klapstein and Colmers, 1993) and the Schaffer collaterals (Colmers *et al.*, 1985, 1987, 1988) are inhibited by NPY. And lastly, NPY may have effects on other dentate gyrus neurons other than granule cells also warrants investigation.

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